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(54) **Title:** ENZYMES AND METHODS FOR DEGRADING CHLORINATED S-TRIAZINES

(57) **Abstract:** The present invention relates to polypeptides for degrading chlorinated s-triazines such as atrazine. The present invention also relates to the use of these polypeptides in the bioremediation of chlorinated s-triazines.

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ENZYMES AND METHODS FOR DEGRADING CHLORINATED S-TRIAZINES

FIELD OF THE INVENTION

The present invention relates to polypeptides for degrading chlorinated s-triazines such as atrazine. The present invention also relates to the use of these polypeptides in the bioremediation of chlorinated s-triazines.

BACKGROUND OF THE INVENTION

Current intensive farming practices are facilitated by the use of effective chemical pest control agents, such as the triazine herbicides. For example, atrazine (6-chloro-N^2-ethyl-N^4-isoproyl-l,3,5-triazine-2,4-diamine) is a highly-effective pre- and post emergence triazine herbicide that has been used extensively for the control of broadleaf weed species since it was first introduced in 1958 (Tomlin, 2006).

Atrazine at environmentally relevant concentrations has been causally linked to endocrine dysfunction in vertebrate species (demasculination of *Xenopus laevis*, for example) (Hayes et al., 2002, 2003 and 2006), and it has been suggested that atrazine may be carcinogenic (Huff, 2002; Huff and Sass, 2007). Additionally, due to their broad specificity, atrazine and related triazine herbicides have the potential to cause environmental damage via their toxic effects on non-target photosynthetic species.

Atrazine is both mobile and persistent in the environment. The environmental half life of atrazine has been estimated to be between four and fifty-seven weeks (Belluck et al., 1991), and atrazine has been detected in both surface and ground waters in several countries (Thurman and Meyer, 1996; van der Meer, 2006; Gavrilescu, 2005).

Several gene/enzyme systems have evolved in prokaryotes that allow the catabolism of the triazine pesticides as sources of carbon and nitrogen. The most thoroughly characterized of these pathways is encoded by the *atzABCDEF* genes from the transmissible pADPl plasmid (Martinez et al., 2001) originally isolated from *Pseudomonas* sp. ADP (Mandelbaum et al., 1995; de Souza et al., 1995). Atrazine and simazine (6-chloro-iV^2,//-diethyl- 1,3,5-triazine-2,4-diamine) (de Souza et al., 1996) are successively dechlorinated and dealkylated by the amidohydrolase family enzymes encoded by *atzA, atzB and atzC* yielding cyanuric acid (de Souza et al., 1996; Boundy-Mills et al., 1997; Sadowsky et al., 1998), which is then mineralised to ammonia and carbon dioxide by the remaining hydrolases in the pathway, encoded by *atzD, atzE* and *atzF* (Fruchey et al., 2003; Cheng et al., 2005; Shapir et al., 2005).

The closest known relative of AtzA is melamine deaminase (TriA; 98 % sequence identity) (Seffernick and Wackett, 2001; Seffernick et al., 2001). Despite

their enormous similarity AtzA and TriA are catalytically distinct, as TriA is a functional deaminase with no detectable dechlorinase, whilst AtzA has no detectable deaminase activity. Previous work has revealed that changes in only two of the nine divergent amino acid residues (Asn328 and Ser331 in AtzA) are directly responsible for orchestrating the selectivity in catalytic target (Raillard et al., 2001). The same study also demonstrated that intermediates between AtzA and TriA possessed activity against substrates not within the range of either parent enzyme.

Bioremediation is an emerging approach to ameliorating the environmental impacts of potentially damaging pesticide residues (Alcalde et al., 2007). One successful bioremediation strategy is that of enzymatic bioremediation, where an isolated or semi-purified enzyme is used to catabolise or modify a toxic pesticide in such a way as to greatly reduce its toxicity (Parales et al., 2002; Sutherland et al., 2004). Enzymatic bioremediation has many advantages over the use of live microorganisms; there is release of GM organisms or intact DNA into the environment, the enzymes used are generally rapid (requiring an application time of only hours) and have a limited, predictable persistence after application (Alcalde et al., 2007). However, the requirements of an enzyme to be employed in bioremediation are somewhat stringent, demanding a high catalytic activity due to limited treatment time during application, a low K_m (for example less than 150 μM) to allow removal of the contaminant to below maximum tolerable limits, lack of dependence upon diffusible co-factors and a generally robust protein toward a range of environmental conditions (pH, temperature, salt concentrations *etc.).*

Although the potentially large environmental footprint of atrazine is concerning, it's continued use in agriculture is desirable. Therefore, there is a need for further methods for eliminating or reducing the potential of atrazine, and other chlorinated s-triazines, for environmental damage.

SUMMARY OF THE INVENTION

The present inventors have identified polypeptides with enhanced ability to degrade atrazine, and other chlorinated s-triazines, when compared to AtzA.

Thus, in a first aspect the present invention provides a substantially purified and/or recombinant polypeptide which has a K_M for atrazine which is less than 150μM.

In an embodiment, the polypeptide has a K_M for atrazine which is less than lOOμM, more preferably less than 75μM.

In a further aspect the present invention provides a substantially purified and/or recombinant polypeptide which has at least a two-fold greater specific activity against atrazine than a polypeptide with a sequence provided as SEQ ID NO: 1.

In an embodiment, the polypeptide has at least a five-fold, more preferably at least a seven-fold, and even more preferably at least a ten-fold, greater specific activity against atrazine than the polypeptide with a sequence provided as SEQ ID NO:1.

In another aspect, the present invention provides a substantially purified and/or recombinant polypeptide comprising amino acids having a sequence which is at least 80% identical to any one or more of SEQ ID NO's 1 to 3, wherein the polypeptide comprises

i) a tyrosine, alanine, serine, glycine, tryptophan, leucine, arginine or histidine at a position corresponding to amino acid number 216 of SEQ ID NO:1, and/or

ii) an alanine, serine, glycine, glutamine, cysteine or aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, and/or

iii) a tyrosine, alanine, serine, glycine, aspartic acid, arginine, valine, leucine, proline, histidine, glutamine or glutamic acid at a position corresponding to amino acid number 219 of SEQ ID NO: 1, and/or

iv) an alanine, serine, glycine, valine, proline, histidine, tryptophan, phenylalanine, tyrosine, leucine, cysteine, aspartic acid, arginine or glutamine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and/or

v) a serine, glycine, valine, proline, histidine, tryptophan, cysteine, glutamic acid, alanine, leucine, tyrosine, arginine, phenylalanine or aspartic acid at a position corresponding to amino acid number 250 of SEQ ID NO:1, and/or

vi) a biologically active fragment of any one of i) to v),

and wherein the polypeptide degrades a chlorinated s-triazine, and wherein if alanine is present at position 216 the polypeptide has at least one of the other amino acid listed in ii) to v), and wherein if alanine is present at position 220 the polypeptide has at least one of the other amino acid listed in i) to iii) or v), and wherein if aspartic acid is present at position 250 the polypeptide has at least one of the other amino acid listed in i) to iv).

In a further aspect, the present invention provides a substantially purified and/or recombinant polypeptide comprising amino acids having a sequence which is at least 80% identical to any one or more of SEQ ID NO's 1 to 3, wherein the polypeptide comprises

i) an alanine, glycine or leucine at a position corresponding to amino acid number 71 of SEQ ID NO:1, and/or

ii) a leucine or tryptophan at a position corresponding to amino acid number 88 of SEQ ID NO: 1, and/or

iii) an alanine, glycine, histidine or tryptophan at a position corresponding to amino acid number 92 of SEQ ID NO:1, and/or

iv) an alanine, serine, phenylalanine or valine at a position corresponding to amino acid number 96 of SEQ ID NO:1, and/or

v) a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:l, or

vi) a biologically active fragment of any one of i) to iv),

and wherein the polypeptide degrades a chlorinated s-triazine, and wherein if leucine is present at position 88 the polypeptide has at least one of the other amino acid listed in i) or iii) to v).

With regard to the two preceding aspects, it is preferred that the chlorinated s-triazine is atrazine, and the polypeptide has at least a two-fold, more preferably at least a five-fold, more preferably at least a seven-fold, and even more preferably at least a ten-fold, greater specific activity against atrazine than the polypeptide provided as SEQ ID NO: 1.

In a further aspect, the present invention provides a substantially purified and/or recombinant polypeptide comprising amino acids having a sequence which is at least 80% identical to any one or more of SEQ ID NO's 1 to 3, wherein the polypeptide comprises

i) a tyrosine, alanine, serine, glycine or histidine at a position corresponding to amino acid number 216 of SEQ ID NO:1, and/or

ii) an alanine, serine, glycine or aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, and/or

iii) a tyrosine, alanine, serine, glycine, aspartic acid, arginine, valine, leucine, proline, histidine or glutamic acid at a position corresponding to amino acid number 219 of SEQ ID NO: 1, and/or

iv) an alanine, serine, glycine, valine, proline, histidine, tryptophan or phenylalanine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and/or

v) a serine, glycine, valine, proline, histidine, tryptophan, cysteine, glutamic acid, alanine, leucine, tyrosine or aspartic acid at a position corresponding to amino acid number 250 of SEQ ID NO:1, and/or

vi) a biologically active fragment of any one of i) to v),

and wherein the polypeptide degrades a chlorinated s-triazine, and wherein if alanine is present at position 216 the polypeptide has at least one of the other amino acids listed in ii) to v), and wherein if alanine is present at position 220 the polypeptide has at least one of the other amino acid listed in i) to iii) or v), and wherein if aspartic acid is present at position 250 the polypeptide has at least one of the other amino acids listed in i) to iv).

In yet another aspect, the present invention provides a substantially purified and/or recombinant polypeptide comprising amino acids having a sequence which is at least 80% identical to any one or more of SEQ ID NO's 1 to 3; wherein the polypeptide comprises

i) an alanine, glycine or leucine at a position corresponding to amino acid number 71 of SEQ ID NO:1, and/or

ii) an alanine, glycine or tryptophan at a position corresponding to amino acid number 92 of SEQ ID NO:1, and/or

iii) an alanine or valine at a position corresponding to amino acid number 96 of SEQ ID NO: 1, and/or

iv) a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:l, or

v) a biologically active fragment of any one of i) to iv),

and wherein the polypeptide degrades a chlorinated s-triazine.

With regard to the two preceding aspects, in a preferred embodiment the chlorinated s-triazine is atrazine, and the polypeptide has at least a five-fold, more preferably at least a seven-fold, and even more preferably at least a ten-fold, greater specific activity against atrazine than the polypeptide provided as SEQ ID NO:1.

In an embodiment, a polypeptide of the invention is fused to at least one other polypeptide sequence. The at least one other polypeptide may be, for example, a polypeptide that enhances the stability of a polypeptide of the present invention, or a polypeptide that assists in the purification of the fusion protein.

In another aspect, the present invention provides an isolated and/or exogenous polynucleotide encoding a polypeptide of the invention.

Preferably, the polynucleotide comprises a sequence which is at least 80% identical to any one or more of SEQ ID NO's 4 to 6. More preferably, the polynucleotide comprises a sequence provide as any one of SEQ ID NO's 4 to 6.

In a further preferred embodiment, the polynucleotide is operably linked to a promoter capable of directing expression of the polynucleotide in a cell.

Also provided is a vector comprising a polynucleotide of the invention.

In another aspect, provided is a host cell comprising a polynucleotide of the invention and/or a vector of the invention. Examples of suitable host cells include, but are not limited to, bacterial, yeast cell, plant and animal cells.

In a further aspect, the present invention provides a transgenic plant or transgenic non-human animal comprising at least one host cell of the invention.

In another aspect, the present invention provides an extract of a host cell of the invention, the plant of the invention and/or the animal of the invention, wherein the extract comprises a polypeptide of the invention.

In yet another aspect, the present invention provides a composition comprising a polypeptide of the invention, a polynucleotide of the invention, a vector of the invention, a host cell of the invention, and/or extract of the invention, and one or more acceptable carriers.

In a further aspect, the present invention provides a method for degrading a chlorinated s-triazine, the method comprising contacting the chlorinated s-triazine with a polypeptide of the invention, a polynucleotide of the invention, a vector of the invention, a host cell of the invention, a plant of the invention, an animal of the invention, an extract of the invention, and/or a composition of the invention.

In an embodiment, the chlorinated s-triazine is in a sample selected from the group consisting of: soil, water, biological material or a combination thereof.

Also provided is a polymeric sponge or foam for degrading a chlorinated s-triazine, the foam or sponge comprising a polypeptide of the invention immobilized on a polymeric porous support.

In another aspect, the present invention provides a method for degrading a chlorinated s-triazine, the method comprising contacting the chlorinated s-triazine with a sponge or foam of the invention.

The present inventors have surprisingly found that polypeptides degrading chlorinated s-triazines display a sigmoidal response. Thus, in a further aspect the present invention provides a method for degrading a chlorinated s-triazine, the method comprising,

i) determining the concentration of the chlorinated s-triazine,

ii) determining the area comprising the chlorinated s-triazine,

iii) calculating the concentration of polypeptide required to degrade the chlorinated s-triazine using a sigmoidal profile,

iv) contacting the chlorinated s-triazine with the polypeptide.

Examples of such sigmoidal profiles are provided in Figure 3 and can be determined by the skilled person using standard techniques.

In a preferred embodiment, the polypeptide is a polypeptide of the invention provided in any form described herein, such as a host cell expressing the polypeptide, a composition comprising the polypeptide etc.

In a preferred embodiment of the previous aspect, the chlorinated s-triazine is atrazine.

In a further aspect, the present invention provides a method of treating toxicity caused by an chlorinated s-triazine in a subject, the method comprising administering to the subject a polypeptide of the invention, a polynucleotide of the invention, a vector of the invention, a host cell of the invention, a plant of the invention, an extract of the invention, and/or a composition of the invention.

Also provided is the use of a polypeptide of the invention, a polynucleotide of the invention, a vector of the invention, a host cell of the invention, a plant of the invention, an extract of the invention and/or a composition of the invention for the manufacture of a medicament for treating toxicity caused by chlorinated s-triazine in a subject.

In another aspect, the present invention provides a method of producing the polypeptide of the invention, the method comprising expressing in a cell, or a cell-free expression system, the polynucleotide of the invention and/or a vector of the invention.

Preferably, the method comprises selecting the polypeptide.

In yet a further aspect, the present invention provides a method for detecting a host cell, the method comprising

i) contacting a cell or a population of cells with a polynucleotide of the invention under conditions which allow uptake of the polynucleotide by the cell(s), and

ii) selecting a host cell by exposing the cells from step i), or progeny cells thereof, to a chlorinated s-triazine.

In an embodiment, the polynucleotide comprises a first open reading frame comprising a polynucleotide of the invention, and a second open reading frame not comprising a polynucleotide of the invention.

In one embodiment, the second open reading frame encodes a polypeptide. In a second embodiment, the second open reading frame encodes a polynucleotide which is not translated. In both instances, it is preferred that the second open reading frame is operably linked to a suitable promoter.

Preferably, the polynucleotide which is not translated encodes a catalytic nucleic acid, a dsRNA molecule, or an antisense molecule.

In a preferred embodiment, the cell is a plant cell.

In a further aspect, the present invention provides a kit for degrading a chlorinated s-triazine, the kit comprising a polypeptide of the invention, a polynucleotide of the invention, a vector of the invention, a host cell of the invention, a plant of the invention, an extract of the invention, a composition of the invention and/or the a sponge or foam of the invention.

As will be apparent, preferred features and characteristics of one aspect of the invention are applicable to many other aspects of the invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1. Structures of the known substrates for atrazine chlorohydrolase (atrazine), imidazolone propionase (4-imidazolone-5-propanoate) and cytosine deaminse (cytosine).

Figure 2. The AtzA homology model. A) *In silico* model of AtzA monomer. The protein secondary structure is indicated (α-helices as helices, and β-strands as arrows), as is the substrate atrazine (spacefilled) docked in the active site. B) Detailed model of atrazine (dark) docked in the active site of AtzA. The residues forming the hydrophobic base (F84, W87 and L88; orange), chloride binding pocket (H66, H68, H243, E246, H276, D327, N328 and S331; green), *N*-ethyl binding pocket (A216, T217, T219, A220 and D250; light blue) and *N*-isopropyl binding pocket (Q71, V92, Q96, E125, D128, M155 and N330; pink) are shown. Those residues that are non-identical in TriA are indicated (*).

Figure 3. Kinetic profiles of the AtzA wild-type enzyme and its improved variants. The last point of each graph represents the solubility limit of the substrate.

KEY TO THE SEQUENCE LISTING

SEQ ID NO:1 - AtzA amino acid sequence.
SEQ ID NO:2 - *N*-isopropyl AtzA consensus sequence of mutants.
SEQ ID NO:3 - *N*-ethyl AtzA consensus sequence of mutants.
SEQ ID NO:4 - Nucleotide sequence encoding AtzA.
SEQ ID NO:5 - Nucleotide sequence encoding *N*-isopropyl AtzA consensus sequence of mutants.
SEQ ID NO:6 - Nucleotide sequence encoding *N*-ethyl AtzA consensus sequence of mutants.
SEQ ID NO's 7 to 11 - Oligonucleotide primers.

DETAILED DESCRIPTION OF THE INVENTION

General Techniques and Definitions

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, immunology, immunohistochemistry, protein chemistry, and biochemistry).

Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan *et al.* (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present).

As used herein, the term "degrades", "degradation" and variations thereof refers the polypeptide of the invention removing at least one chloride from the chlorinated s-triazine.

As used herein the terms "treating", "treat" or "treatment" include administering a therapeutically effective amount of a polypeptide of the invention, or a polynucleotide encoding therefor, sufficient to reduce or eliminate at least one symptom of toxicity caused by an chlorinated s-triazine.

The term "biological material" is used herein in its broadest sense to include any product of biological origin. Such products include, but are not restricted to, food products for humans and animal feeds. The products include liquid media including water and liquid foodstuffs such as milk, as well as semi-solid foodstuffs such as yoghurt and the like. The present invention also extends to solid foodstuffs, particularly animal feeds. In an embodiment, it is preferred that the biological material is plant material such as, but not limited to, sugar cane, canola seeds, wheat seeds, barley seeds, sorghum seeds, rice, corn, pineapples, or cotton seeds.

As used herein, the phrase "at a position corresponding to amino acid number" refers to the relative position of the amino acid compared to surrounding amino acids. For example, in some embodiments a polypeptide of the invention may have deletional or substitutional mutations which alters the relative positioning of the amino acid when aligned against, for example, SEQ ID NO:1.

As used herein, the term "extract" refers to any portion of a host cell, plant or non-human transgenic animal of the invention. The portion may be a whole entity such as a seed of a plant, or obtained by at least partial homogenization and/or purification.

In accordance with standard practice, K_m refers to the Michaelis constant which is the substrate concentration at which the enzyme functions at one half of its maximal rate. Furthermore, in accordance with standard practice the specific activity is the rate per unit enzyme at a given concentration of substrate. In a preferred embodiment, the substrate is atrazine and the concentration is 23μM.

Chlorinated s-Triazines

S-triazine (1,3,5-triazine) is an organic chemical compound whose chemical structure has a six-membered heterocyclic aromatic ring consisting of three carbon atoms and three nitrogen atoms. The atoms in triazine rings are analogous to those in benzene rings.

Chlorinated s-triazines comprise at least one chloride. Examples of chlorinated s-triazines include, but are not limited to, atrazine (6-chloro-*N*-ethyl-*N*'-(l-methylethyl)-l,3,5-triazine-2,4-diamine) (see Figure 1), chlorazine (6-chloro-*N*,*N*,*N*yV'-tetraethyl-1,3,5-triazine-2,4-diamine), cyanazine (2-[[4-chloro-6-(ethylamino)-l ,3,5-triazin-2-yl]amino]-2-methylpropanenitrile), cyprazine (6-chloro-*N*-cyclopropyl-*N*'-(l-methylethyl)-l,3,5-triazine-2,4-diamine), eglinazine *(N-[4*-chloro-6-(ethylamino)- 1,3,5-triazin-2-yl]glycine), ipazine (6-chloro-*N*,*N*-diethyl-*N'*-(l-methylethyl)-l,3,5-triazine-2,4-diamine), mesoprazine (6-chloro-*N*-(3-methoxypropyl)-*N*'-(l-methylethyl)-l,3,5-triazine-2,4-diamine), procyazine (2-[[4-chloro-6-(cyclopropylamino)-l,3,5-triazin-2-yl]amino]-2-methylpropanenitrile), proglinazine *N*-[4-chloro-6-[(l-methylethyl)amino]-l,3,5-triazin-2-yl]glycine), propazine (6-chloro-*N*,*N*'-bis(l-methylethyl)-l,3,5-triazine-2,4-diamine), sebuthylazine (6-chloro-*N*-ethyl-*N*'-(l-methylpropyl)-l,3,5-triazine-2,4-diamine, simazine (6-chloro-*N*,*N*'-diethyl-l,3,5-triazine-2,4-diamine), terbuthylazine (6-chloro-*N*-(l,l-dimethylethyl)- *N*'-ethyl-l,3,5-triazine-2,4-diamine) and trietazine (6-chloro-*N*,*N*,*N*'-triethyl-l,3,5-triazine-2,4-diamine), as well as products of chloro-s-triazine dealkylation (including atrazine dealkylation such as desethyl atrazine and desisopropyl atrazine).

In a particularly preferred embodiment, the chlorinated s-triazine is atrazine, simazine, desethyl atrazine or desisopropyl atrazine, even more preferably the chlorinated s-triazine is atrazine.

Polypeptides

By "substantially purified" or "purified" we mean a polypeptide that has been separated from one or more lipids, nucleic acids, other polypeptides, or other contaminating molecules with which it is associated in its native state. It is preferred that the substantially purified polypeptide is at least 60% free, more preferably at least

75% free, and more preferably at least 90% free from other components with which it is naturally associated. However, at present there is no evidence that the polypeptides of the invention exist in nature.

The term "recombinant" in the context of a polypeptide refers to the polypeptide when produced by a cell, or in a cell-free expression system, in an altered amount or at an altered rate compared to its native state. In one embodiment the cell is a cell that does not naturally produce the polypeptide. However, the cell may be a cell which comprises a non-endogenous gene that causes an altered, preferably increased, amount of the polypeptide to be produced. A recombinant polypeptide of the invention includes polypeptides which have not been separated from other components of the transgenic (recombinant) cell, or cell-free expression system, in which it is produced, and polypeptides produced in such cells or cell-free systems which are subsequently purified away from at least some other components.

The terms "polypeptide" and "protein" are generally used interchangeably and refer to a single polypeptide chain which may or may not be modified by addition of non-amino acid groups. It would be understood that such polypeptide chains may associate with other polypeptides or proteins or other molecules such as co-factors. The terms "proteins" and "polypeptides" as used herein also include variants, mutants, biologically active fragments, modifications, analogous and/or derivatives of the polypeptides described herein.

The % identity of a polypeptide is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. The query sequence is at least 25 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 25 amino acids. More preferably, the query sequence is at least 50 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 50 amino acids. More preferably, the query sequence is at least 100 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 100 amino acids. Even more preferably, the query sequence is at least 250 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 250 amino acids. Even more preferably, the GAP analysis aligns the two sequences over their entire length.

As used herein a "biologically active fragment" is a portion of a polypeptide as described herein which maintains a defined activity of the full-length polypeptide. Biologically active fragments can be any size as long as they maintain the defined activity. Preferably, biologically active fragments are at least 100 amino acids in length.

With regard to a defined polypeptide, it will be appreciated that % identity figures higher than those provided above will encompass preferred embodiments.

Thus, where applicable, in light of the minimum % identity figures, it is preferred that the polypeptide comprises an amino acid sequence which is at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, more preferably at least 99.1%, more preferably at least 99.2%, more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least 99.7%, more preferably at least 99.8%, and even more preferably at least 99.9% identical to the relevant nominated SEQ ID NO.

Amino acid sequence mutants of a polypeptide described herein can be prepared by introducing appropriate nucleotide changes into a nucleic acid defined herein, or by *in vitro* synthesis of the desired polypeptide. Such mutants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final polypeptide product possesses the desired characteristics.

Mutant (altered) polypeptides can be prepared using any technique known in the art. For example, a polynucleotide described herein can be subjected to *in vitro* mutagenesis. Such *in vitro* mutagenesis techniques may include sub-cloning the polynucleotide into a suitable vector, transforming the vector into a "mutator" strain such as the *E. coli* XL-I red (Stratagene) and propagating the transformed bacteria for a suitable number of generations. In another example, the polynucleotides of the invention are subjected to DNA shuffling techniques as broadly described by Harayama (1998). Products derived from mutated/altered DNA can readily be screened using techniques described herein to determine if they are able to confer the desired phenotype such as enhanced activity and/or altered substrate specificity.

In designing amino acid sequence mutants, the location of the mutation site and the nature of the mutation will depend on characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting other residues adjacent to the located site.

Amino acid sequence deletions generally range from about 1 to 15 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues.

Substitution mutants have at least one amino acid residue in the polypeptide molecule removed and a different residue inserted in its place. The sites of greatest

interest for substitutional mutagenesis include sites identified as important for function. Other sites of interest are those in which particular residues obtained from various strains or species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are preferably substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1.

Table 1 - Exemplary substitutions.

Original Residue	**Exemplary Substitutions**
Ala (A)	val; leu; ile; gly
Arg (R)	lys
Asn (N)	gln; his
Asp (D)	glu
Cys (C)	ser
Gln (Q)	asn; his
Glu (E)	asp
Gly (G)	pro, ala
His (H)	asn; gln
Ile (I)	leu; val; ala
Leu (L)	ile; val; met; ala; phe
Lys (K)	arg
Met (M)	leu; phe
Phe (F)	leu; val; ala
Pro (P)	gly
Ser (S)	thr
Thr (T)	ser
Trp (W)	tyr
Tyr (Y)	trp; phe
Val (V)	ile; leu; met; phe; ala

In an embodiment, the polypeptide comprises a glycine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a valine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a histidine at a position

corresponding to amino acid number 220 of SEQ ID NO:1, and a aspartic acid at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a proline at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a arginine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another embodiment, the polypeptide comprises a glycine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glutamic acid at a position corresponding to amino acid number 219 of SEQ ID NO:1, a tyrosine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a proline at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a proline at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a cysteine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a proline at a position corresponding to amino acid number 219 of SEQ ID NO:1, a tyrosine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a alanine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a histidine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glutamine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a phenylalanine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a serine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a tryptophan at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a arginine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another embodiment, the polypeptide comprises a serine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a serine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a glutamine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a cysteine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a phenylalanine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a glycine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 219 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a aspartic acid at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a tyrosine at a position corresponding to amino acid number 219 of SEQ ID NO.l, a leucine at a position corresponding to amino acid number 220 of SEQ ID NO.l, and a arginine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a histidine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a tyrosine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a valine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a serine at a position

corresponding to amino acid number 217 of SEQ ID NO:1, a glutamine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a glutamine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glycine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a histidine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a cysteine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glutamic acid at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a leucine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a glutamine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a valine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a arginine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a arginine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a alanine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another embodiment, the polypeptide comprises a glycine at a position corresponding to amino acid number 71 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 88 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 92 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 96 of SEQ ID NO:1, and a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO: 1.

In another embodiment, the polypeptide comprises a glycine at a position corresponding to amino acid number 71 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 88 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 92 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 96 of SEQ ID NO:1, and a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO: 1.

In a further embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 71 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 88 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 92 of SEQ ID NO:1, a serine at a position

corresponding to amino acid number 96 of SEQ ID NO:1, and a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a leucine at a position corresponding to amino acid number 71 of SEQ ID NO:1, a tryptophan at a position corresponding to amino acid number 88 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 92 of SEQ ID NO:1, a phenylalanine at a position corresponding to amino acid number 96 of SEQ ID NO:1, and a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:1.

In another embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 71 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 88 of SEQ ID NO:1, a tryptophan at a position corresponding to amino acid number 92 of SEQ ID NO:1, a phenylalanine at a position corresponding to amino acid number 96 of SEQ ID NO:1, and a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:1.

In yet another embodiment, the polypeptide comprises a glycine at a position corresponding to amino acid number 71 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 88 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 92 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 96 of SEQ ID NO:1, and a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:1.

In a preferred embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a proline at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a cysteine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 219 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glutamic acid at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 219 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a alanine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a serine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a arginine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glutamic acid at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a valine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glycine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a leucine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a tryptophan at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a serine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a proline at a position corresponding to amino acid number 219 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a tryptophan at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a tyrosine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a serine at a

position corresponding to amino acid number 217 of SEQ ID NO:1, a proline at a position corresponding to amino acid number 219 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a proline at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a tyrosine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a histidine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a tyrosine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a valine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a serine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a proline at a position corresponding to amino acid number 219 of SEQ ID NO:1, a phenylalanine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glycine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a glycine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glycine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a tyrosine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a serine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a valine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a histidine at a

position corresponding to amino acid number 220 of SEQ ID NO:1, and a glycine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glutamic acid at a position corresponding to amino acid number 219 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a aspartic acid at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a glycine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a valine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a tryptophan at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a histidine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glutamic acid at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a glycine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glutamic acid at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a histidine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glutamic acid at a position corresponding to amino acid number 219 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a serine at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the' polypeptide comprises a glycine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glutamic acid at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a serine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a serine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a aspartic acid at a position corresponding to amino acid number 250 of SEQ ID NO: 1.

In another preferred embodiment, the polypeptide comprises a tyrosine at a position corresponding to amino acid number 216 of SEQ ID NO:1, a aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, a threonine at a position corresponding to amino acid number 219 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and a glutamine at a position corresponding to amino acid number 250 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a alanine at a position corresponding to amino acid number 71 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 88 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 92 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 96 of SEQ ID NO:1, and a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a glycine at a position corresponding to amino acid number 71 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 88 of SEQ ID NO:1, a tryptophan at a position corresponding to amino acid number 92 of SEQ ID NO:1, a valine at a position corresponding to amino acid number 96 of SEQ ID NO:1, and a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:1.

In another preferred embodiment, the polypeptide comprises a leucine at a position corresponding to amino acid number 71 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 88 of SEQ ID NO:1, a glycine at a position corresponding to amino acid number 92 of SEQ ID NO:1, a alanine at a position corresponding to amino acid number 96 of SEQ ID NO: 1, and a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:1.

In a preferred embodiment, the polypeptide does not comprises an aspartic acid at a position corresponding to amino acid number 328 of SEQ ID NO:1, a cysteine at a position corresponding to amino acid number 331 of SEQ ID NO:1, a isoleucine at a position corresponding to amino acid number 217 of SEQ ID NO:1, and/or a proline at a position corresponding to amino acid number 219 of SEQ ID NO:1.

Preferably, if not specified otherwise, at a given amino acid position the polypeptide comprises an amino acid as found at the corresponding position of the polypeptide provided as SEQ ID NO:1.

In yet a further preferred embodiment, if the amino acid at a position corresponding to amino acid number 217 of SEQ ID NO:1 is not as defined herein it is preferably threonine.

In yet a further preferred embodiment, if the amino acid at a position corresponding to amino acid number 219 of SEQ ID NO:1 is not as defined herein it is preferably threonine.

In yet a further preferred embodiment, if the amino acid at a position corresponding to amino acid number 71 of SEQ ID NO:1 is not as defined herein it is preferably glutamic acid.

In a preferred embodiment, a leucine is present at a position corresponding to amino acid number 88 of SEQ ID NO: 1.

In yet a further preferred embodiment, if the amino acid at a position corresponding to amino acid number 92 of SEQ ID NO:1 is not as defined herein it is preferably valine.

In yet a further preferred embodiment, if the amino acid at a position corresponding to amino acid number 96 of SEQ ID NO: 1 is not as defined herein it is preferably glutamine.

In yet a further preferred embodiment, if the amino acid at a position corresponding to amino acid number 330 of SEQ ID NO:1 is not as defined herein it is preferably asparagine.

In a preferred embodiment, the polypeptide comprises a histidine at a position corresponding to amino acid number 66 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 68 of SEQ ID NO:1, a histidine at a position corresponding to amino acid number 243 of SEQ ID NO:1, a glutamic acid at a position corresponding to amino acid number 246 of SEQ ID NO:1, at histidine a position corresponding to amino acid number 276 of SEQ ID NO:1, an aspartic acid at a position corresponding to amino acid number 327 of SEQ ID NO:1, tryptophan at a position corresponding to amino acid number 87 of SEQ ID NO:1, a leucine at a position corresponding to amino acid number 88 of SEQ ID NO:1, an asparagine at a position corresponding to amino acid number 328 of SEQ ID NO:1 and/or a serine at a position corresponding to amino acid number 331 of SEQ ID NO: 1.

In yet a further embodiment, if asparagine is at a position corresponding to amino acid number 330 of SEQ ID NO:1 then valine is present at a position corresponding to amino acid number 92 of SEQ ID NO:1.

If an amino acid at a nominated site is inconsistent with an amino acid substitution provided in Table 1, the nominated amino acid is preferred.

Furthermore, if desired, unnatural amino acids or chemical amino acid analogues can be introduced as a substitution or addition into a polypeptides described

herein. Such amino acids include, but are not limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogues in general.

Also included within the scope of the invention are polypeptides of the present invention which are differentially modified during or after synthesis, e.g., by biotinylation, benzylation, glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. These modifications may serve to increase the stability and/or bioactivity of the polypeptide.

Polypeptides described herein can be produced in a variety of ways, including production and recovery of natural polypeptides, production and recovery of recombinant polypeptides, and chemical synthesis of the polypeptides. In one embodiment, an isolated polypeptide of the present invention is produced by culturing a cell capable of expressing the polypeptide under conditions effective to produce the polypeptide, and recovering the polypeptide. A preferred cell to culture is a recombinant cell of the present invention. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit polypeptide production. An effective medium refers to any medium in which a cell is cultured to produce a polypeptide of the present invention. Such medium typically comprises an aqueous medium having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri, plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Polynucleotides

By an "isolated polynucleotide", including DNA, RNA, or a combination of these, single or double stranded, in the sense or antisense orientation or a combination of both, dsRNA or otherwise, we mean a polynucleotide which is at least partially separated from the polynucleotide sequences with which it is associated or linked in its native state. Preferably, the isolated polynucleotide is at least 60% free, preferably

at least 75% free, and most preferably at least 90% free from other components with which they are naturally associated. Furthermore, the term "polynucleotide" is used interchangeably herein with the term "nucleic acid".

The term "exogenous" in the context of a polynucleotide refers to the polynucleotide when present in a cell, or in a cell-free expression system, in an altered amount compared to its native state. In one embodiment, the cell is a cell that does not naturally comprise the polynucleotide. However, the cell may be a cell which comprises a non-endogenous polynucleotide resulting in an altered, preferably increased, amount of production of the encoded polypeptide. An exogenous polynucleotide of the invention includes polynucleotides which have not been separated from other components of the transgenic (recombinant) cell, or cell-free expression system, in which it is present, and polynucleotides produced in such cells or cell-free systems which are subsequently purified away from at least some other components.

The % identity of a polynucleotide is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. Unless stated otherwise, the query sequence is at least 45 nucleotides in length, and the GAP analysis aligns the two sequences over a region of at least 45 nucleotides. Preferably, the query sequence is at least 150 nucleotides in length, and the GAP analysis aligns the two sequences over a region of at least 150 nucleotides. More preferably, the query sequence is at least 300 nucleotides in length and the GAP analysis aligns the two sequences over a region of at least 300 nucleotides. Even more preferably, the GAP analysis aligns the two sequences over their entire length.

With regard to the defined polynucleotides, it will be appreciated that % identity figures higher than those provided above will encompass preferred embodiments. Thus, where applicable, in light of the minimum % identity figures, it is preferred that a polynucleotide of the invention comprises a sequence which is at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, more preferably at least 99.1%, more preferably at least 99.2%, more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least 99.7%, more preferably at least 99.8%, and even more preferably at least 99.9% identical to the relevant nominated SEQ ID NO.

The present invention also relates to a polynucleotide which hybridizes under stringent conditions to a polynucleotide encoding one or more of SEQ ID NO:4, SEQ

ID NO:5 and/or SEQ ID NO:6 and which encodes a polypeptide of the invention. The term "stringent hybridization conditions" or "stringent conditions" and the like as used herein refers to parameters with which the art is familiar, including the variation of the hybridization temperature with length of an polynucleotide or oligonucleotide. Nucleic acid hybridization parameters may be found in references which compile such methods, Sambrook, et al., *{supra),* and Ausubel, et al., *{supra).* For example, stringent hybridization conditions, as used herein, can refer to hybridization at 65°C in hybridization buffer (3.5xSSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5 mM NaH_2PO_4 (pH7), 0.5% SDS, 2 mM EDTA) and washing twice in 0.2xSSC, 0.1% SDS at 65^0C; with each wash step being about 30 min.

Polynucleotides of the present invention may possess, when compared to molecules provided herewith, one or more mutations which are deletions, insertions, or substitutions of nucleotide residues. Mutants can be either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis on the nucleic acid).

Usually, monomers of a polynucleotide are linked by phosphodiester bonds or analogs thereof. Analogs of phosphodiester linkages include: phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate and phosphoramidate.

Recombinant Vectors

One embodiment of the present invention includes a recombinant vector, which comprises at least one isolated polynucleotide encoding a polypeptide as described herein, inserted into any vector capable of delivering the polynucleotide molecule into a host cell. Such a vector contains heterologous polynucleotide sequences, that is polynucleotide sequences that are not naturally found adjacent to polynucleotide molecules of the present invention and that preferably are derived from a species other than the species from which the polynucleotide molecule(s) are derived. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a transposon (such as described in US 5,792,294), a virus or a plasmid.

One type of recombinant vector comprises the polynucleotide(s) operably linked to an expression vector. The phrase operably linked refers to insertion of a polynucleotide molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified polynucleotide molecule. Preferably, the expression vector is also capable of replicating within the host cell. Expression

vectors can be either prokaryotic or eukaryotic, and are typically viruses or plasmids. Expression vectors include any vectors that function (i.e., direct gene expression) in recombinant cells, including in bacterial, fungal, endoparasite, arthropod, animal, and plant cells. Vectors of the invention can also be used to produce the polypeptide in a cell-free expression system, such systems are well known in the art.

"Operably linked" as used herein refers to a functional relationship between two or more nucleic acid (e.g., DNA) segments. Typically, it refers to the functional relationship of transcriptional regulatory element to a transcribed sequence. For example, a promoter is operably linked to a coding sequence, such as a polynucleotide defined herein, if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell and/or in a cell-free expression system. Generally, promoter transcriptional regulatory elements that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are *cis*-acting. However, some transcriptional regulatory elements, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

In particular, expression vectors of the present invention contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of polynucleotide molecules of the present invention. In particular, recombinant molecules of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in bacterial, yeast, arthropod, nematode, plant or animal cells, such as, but not limited to, *tac, lac, ara trp, trc, oxy-pro, omp/lpp, rrnB,* bacteriophage lambda, bacteriophage T7, Y*llac,* bacteriophage T3, bacteriophage SP6, bacteriophage SPOl, metallothionein, alpha-mating factor, Pichia alcohol oxidase, alphavirus subgenomic promoters (such as Sindbis virus subgenomic promoters), antibiotic resistance gene, baculovirus, *Heliothis zea* insect virus, vaccinia virus, herpesvirus, raccoon poxvirus, other poxvirus, adenovirus, cytomegalovirus (such as intermediate early promoters), simian virus 40, retrovirus, actin, retroviral long terminal repeat, Rous sarcoma virus, heat

shock, phosphate and nitrate transcription control sequences as well as other sequences capable of controlling gene expression in prokaryotic or eukaryotic cells.

Host Cells

Another embodiment of the present invention includes a host cell transformed with one or more recombinant molecules described herein or progeny cells thereof. Transformation of a polynucleotide molecule into a cell can be accomplished by any method by which a polynucleotide molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. Transformed polynucleotide molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

Suitable host cells to transform include any cell that can be transformed with a polynucleotide of the present invention. Host cells of the present invention either can be endogenously (i.e., naturally) capable of producing polypeptides described herein or can be capable of producing such polypeptides after being transformed with at least one polynucleotide molecule as described herein. Host cells of the present invention can be any cell capable of producing at least one protein defined herein, and include bacterial, fungal (including yeast), parasite, nematode, arthropod, animal and plant cells. Examples of host cells include *Salmonella, Escherichia, Bacillus, Listeria, Saccharomyces, Spodoptera, Mycobacteria, Trichoplusia,* BHK (baby hamster kidney) cells, MDCK cells, CRFK cells, CV-I cells, COS (e.g., COS-7) cells, and Vero cells. Further examples of host cells are *E. coli,* including *E. coli* K-12 derivatives; *Salmonella typhi; Salmonella typhimurium,* including attenuated strains; *Spodoptera frugiperda; Trichoplusia ni;* and non-tumorigenic mouse myoblast G8 cells (e.g., ATCC CRL 1246). Particularly preferred host cells are bacterial cells or plant cells.

Recombinant DNA technologies can be used to improve expression of a transformed polynucleotide molecule by manipulating, for example, the number of copies of the polynucleotide molecule within a host cell, the efficiency with which those polynucleotide molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of polynucleotide molecules of the present invention include, but are not limited to, operatively linking polynucleotide molecules to high-copy number plasmids, integration of the

polynucleotide molecule into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of polynucleotide molecules of the present invention-to correspond to the codon usage of the host cell, and the deletion of sequences that destabilize transcripts.

Transgenic Plants

Plants contemplated for use in the practice of the present invention include both monocotyledons and dicotyledons. Target plants include, but are not limited to, the following: cereals (for example, wheat, barley, rye, oats, rice, maize, sorghum and related crops); beet (sugar beet and fodder beet); pomes, stone fruit and soft fruit (apples, pears, plums, peaches, almonds, cherries, strawberries, raspberries and black¬ berries); leguminous plants (beans, lentils, peas, soybeans); oil plants (peanut, rape, mustard, poppy, olives, sunflowers, coconut, castor oil plants, cocoa beans, groundnuts); cucumber plants (marrows, cucumbers, melons); fibre plants (cotton, flax, hemp, jute); citrus fruit (oranges, lemons, grapefruit, mandarins); vegetables (spinach, lettuce, asparagus, cabbages, carrots, onions, tomatoes, potatoes, paprika); lauraceae (avocados, cinnamon, camphor); or plants such as tobacco, nuts, coffee, sugar cane, tea, vines, hops, turf, bananas and natural rubber plants, as well as ornamentals (flowers, shrubs, broad-leaved trees and evergreens, such as conifers). Crops frequently effected by *Aspergillus sp.* infection which are target plants of the invention include, but are not limited to, cereals (maize, sorghum, pearl millet, rice, wheat), oilseeds (peanut, soybean, sunflower, cotton), spices (chile peppers, black pepper, coriander, turmeric, ginger), and tree nuts (almond, pistachio, walnut, coconut). In a preferred embodiment, the plant is selected from sugar, cotton, corn, sorghum, pineapple, conifers such as Christmas trees, eucalypts, wheat, oats, barley, rice and canola.

The term "plant" as used herein as a noun refers to a whole plants such as, for example, a plant growing in a field for commercial wheat production. A "plant part" refers to vegetative structures (for example, leaves, stems), roots, floral organs/structures, seed (including embryo, endosperm, and seed coat), plant tissue (for example, vascular tissue, ground tissue, and the like), cells and progeny of the same.

Transgenic plants, as defined in the context of the present invention include plants (as well as parts and cells of said plants) and their progeny which have been genetically modified using recombinant techniques to cause production of at least one polypeptide of the present invention in the desired plant or plant organ. Transgenic

plants can be produced using techniques known in the art, such as those generally described in A. Slater et al., Plant Biotechnology - The Genetic Manipulation of Plants, Oxford University Press (2003), and P. Christou and H. Klee, Handbook of Plant Biotechnology, John Wiley and Sons (2004).

A "transgenic plant" refers to a plant that contains a gene construct ("transgene") not found in a wild-type plant of the same species, variety or cultivar. A "transgene" as referred to herein has the normal meaning in the art of biotechnology and includes a genetic sequence which has been produced or altered by recombinant DNA or RNA technology and which has been introduced into the plant cell. The transgene may include genetic sequences derived from a plant cell. Typically, the transgene has been introduced into the plant by human manipulation such as, for example, by transformation but any method can be used as one of skill in the art recognizes.

In a preferred embodiment, the transgenic plants are homozygous for each and every gene that has been introduced (transgene) so that their progeny do not segregate for the desired phenotype. The transgenic plants may also be heterozygous for the introduced transgene(s), such as, for example, in F1 progeny which have been grown from hybrid seed. Such plants may provide advantages such as hybrid vigour, well known in the art.

A polynucleotide of the present invention may be expressed constitutively in the transgenic plants during all stages of development. Depending on the use of the plant or plant organs, the polypeptides may be expressed in a stage-specific manner. Furthermore, the polynucleotides may be expressed tissue-specifically.

Regulatory sequences which are known or are found to cause expression of a gene encoding a polypeptide of interest in plants may be used in the present invention. The choice of the regulatory sequences used depends on the target plant and/or target organ of interest. Such regulatory sequences may be obtained from plants or plant viruses, or may be chemically synthesized. Such regulatory sequences are well known to those skilled in the art.

A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, supp. 1987; Weissbach and Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989; and Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic Publishers, 1990. Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or

developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

A number of constitutive promoters that are active in plant cells have been described. Suitable promoters for constitutive expression in plants include, but are not limited to, the cauliflower mosaic virus (CaMV) 35S promoter, the Figwort mosaic virus (FMV) 35S, the sugarcane bacilliform virus promoter, the commelina yellow mottle virus promoter, the light-inducible promoter from the small subunit of the ribulose-l,5-bis-phosphate carboxylase, the rice cytosolic triosephosphate isomerase promoter, the adenine phosphoribosyltransferase promoter of *Arabidopsis,* the rice actin 1 gene promoter, the mannopine synthase and octopine synthase promoters, the Adh promoter, the sucrose synthase promoter, the R gene complex promoter, and the chlorophyll α,β binding protein gene promoter. These promoters have been used to create DNA vectors that have been expressed in plants; see, e.g., PCT publication WO 8402913. All of these promoters have been used to create various types of plant-expressible recombinant DNA vectors.

For the purpose of expression in source tissues of the plant, such as the leaf, seed, root or stem, it is preferred that the promoters utilized in the present invention have relatively high expression in these specific tissues. For this purpose, one may choose from a number of promoters for genes with tissue- or cell-specific or -enhanced expression. Examples of such promoters reported in the literature include the chloroplast glutamine synthetase GS2 promoter from pea, the chloroplast fructose-1,6-biphosphatase promoter from wheat, the nuclear photosynthetic ST-LS 1 promoter from potato, the serine/threonine kinase promoter and the glucoamylase (CHS) promoter from *Arabidopsis thaliana.* Also reported to be active in photosynthetically active tissues are the ribulose-l,5-bisphosphate carboxylase promoter from eastern larch *(Larix laricina),* the promoter for the Cab gene, Cab6, from pine, the promoter for the Cab-1 gene from wheat, the promoter for the Cab-1 gene from spinach, the promoter for the Cab IR gene from rice, the pyruvate, orthophosphate dikinase (PPDK) promoter from *Zea mays,* the promoter for the tobacco Lhcbl*2 gene, the *Arabidopsis thaliana* Suc2 sucrose-H^{30} symporter promoter, and the promoter for the thylakoid membrane protein genes from spinach (PsaD, PsaF, PsaE, PC, FNR, AtpC, AtpD, Cab, RbcS).

Other promoters for the chlorophyll α,β-binding proteins may also be utilized in the present invention, such as the promoters for LhcB gene and PsbP gene from white mustard *(Sinapis alba).* A variety of plant gene promoters that are regulated in response to environmental, hormonal, chemical, and/or developmental signals, also can be used for expression of RNA-binding protein genes in plant cells, including

promoters regulated by (1) heat, (2) light (e.g., pea RbcS-3A promoter, maize RbcS promoter); (3) hormones, such as abscisic acid, (4) wounding (e.g., Wunl); or (5) chemicals, such as methyl jasminate, salicylic acid, steroid hormones, alcohol, Safeners (WO 97/06269), or it may also be advantageous to employ (6) organ-specific promoters.

For the purpose of expression in sink tissues of the plant, such as the tuber of the potato plant, the fruit of tomato, or the seed of soybean, canola, cotton, *Zea mays*, wheat, rice, and barley, it is preferred that the promoters utilized in the present invention have relatively high expression in these specific tissues. A number of promoters for genes with tuber-specific or -enhanced expression are known, including the class I patatin promoter, the promoter for the potato tuber ADPGPP genes, both the large and small subunits, the sucrose synthase promoter, the promoter for the major tuber proteins including the 22 kD protein complexes and proteinase inhibitors, the promoter for the granule bound starch synthase gene (GBSS), and other class I and II patatins promoters. Other promoters can also be used to express a protein in specific tissues, such as seeds or fruits. The promoter for β-conglycinin or other seed-specific promoters such as the napin and phaseolin promoters, can be used. A particularly preferred promoter for *Zea mays* endosperm expression is the promoter for the glutelin gene from rice, more particularly the Osgt-1 promoter. Examples of promoters suitable for expression in wheat include those promoters for the ADPglucose pyrosynthase (ADPGPP) subunits, the granule bound and other starch synthase, the branching and debranching enzymes, the embryogenesis-abundant proteins, the gliadins, and the glutenins. Examples of such promoters in rice include those promoters for the ADPGPP subunits, the granule bound and other starch synthase, the branching enzymes, the debranching enzymes, sucrose synthases, and the glutelins. A particularly preferred promoter is the promoter for rice glutelin, Osgt-1 gene. Examples of such promoters for barley include those for the ADPGPP subunits, the granule bound and other starch synthase, the branching enzymes, the debranching enzymes, sucrose synthases, the hordeins, the embryo globulins, and the aleurone specific proteins.

Root specific promoters may also be used. An example of such a promoter is the promoter for the acid chitinase gene. Expression in root tissue could also be accomplished by utilizing the root specific subdomains of the CaMV 35S promoter that have been identified.

The 5' non-translated leader sequence can be derived from the promoter selected to express the heterologous gene sequence of the polynucleotide of the present invention, and can be specifically modified if desired so as to increase translation of mRNA. For a review of optimizing expression of transgenes, see

Koziel et al. (1996). The 5' non-translated regions can also be obtained from plant viral RNAs (Tobacco mosaic virus, Tobacco etch virus, Maize dwarf mosaic virus, Alfalfa mosaic virus, among others) from suitable eukaryotic genes, plant genes (wheat and maize chlorophyll a/b binding protein gene leader), or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. The leader sequence could also be derived from an unrelated promoter or coding sequence. Leader sequences useful in context of the present invention comprise the maize Hsp70 leader (U.S. 5,362,865 and U.S. 5,859,347), and the TMV omega element.

The termination of transcription is accomplished by a 3' non-translated DNA sequence operably linked in the chimeric vector to the polynucleotide of interest. The 3' non-translated region of a recombinant DNA molecule contains a polyadenylation signal that functions in plants to cause the addition of adenylate nucleotides to the 3' end of the RNA. The 3' non-translated region can be obtained from various genes that are expressed in plant cells. The nopaline synthase 3' untranslated region, the 3' untranslated region from pea small subunit Rubisco gene, the 3' untranslated region from soybean 7S seed storage protein gene are commonly used in this capacity. The 3' transcribed, non-translated regions containing the polyadenylate signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes are also suitable.

Four general methods for direct delivery of a gene into cells have been described: (1) chemical methods (Graham et al., 1973); (2) physical methods such as microinjection (Capecchi, 1980); electroporation (see, for example, WO 87/06614, US 5,472,869, 5,384,253, WO 92/09696 and WO 93/21335); and the gene gun (see, for example, US 4,945,050 and US 5,141,131); (3) viral vectors (Clapp, 1993; Lu et al., 1993; Eglitis et al., 1988); and (4) receptor-mediated mechanisms (Curiel et al., 1992; Wagner et al., 1992).

Acceleration methods that may be used include, for example, microprojectile bombardment and the like. One example of a method for delivering transforming nucleic acid molecules to plant cells is microprojectile bombardment. This method has been reviewed by Yang et al., Particle Bombardment Technology for Gene Transfer, Oxford Press, Oxford, England (1994). Non-biological particles (microprojectiles) are coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like. A particular advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly transforming monocots, is that neither the isolation of protoplasts, nor the susceptibility of *Agrobacterium* infection are required. An illustrative embodiment of a method for delivering DNA into *Zea*

mays cells by acceleration is a biolistics α-particle delivery system, that can be used to propel particles coated with DNA through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with corn cells cultured in suspension. A particle delivery system suitable for use with the present invention is the helium acceleration PDS-1000/He gun, available from Bio-Rad Laboratories.

For the bombardment, cells in suspension may be concentrated on filters. Filters containing the cells to be bombarded are positioned at an appropriate distance below the microprojectile stopping plate. If desired, one or more screens are also positioned between the gun and the cells to be bombarded.

Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the microprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more foci of cells transiently expressing a marker gene. The number of cells in a focus that express the exogenous gene product 48 hours post-bombardment often range from one to ten and average one to three.

In bombardment transformation, one may optimize the pre-bombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters for bombardment are important in this technology. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature embryos.

In another alternative embodiment, plastids can be stably transformed. Methods disclosed for plastid transformation in higher plants include particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination (U.S. 5, 451,513, U.S. 5,545,818, U.S. 5,877,402, U.S. 5,932479, and WO 99/05265).

Accordingly, it is contemplated that one may wish to adjust various aspects of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance, and helium pressure. One may also minimize the trauma reduction factors by modifying conditions that influence the physiological state of the

recipient cells and that may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art in light of the present disclosure.

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium-mediated* plant integrating vectors to introduce DNA into plant cells is well known in the art (see, for example, US 5,177,010, US 5,104,310, US 5,004,863, US 5,159,135). Further, the integration of the T-DNA is a relatively precise process resulting in few rearrangements. The region of DNA to be transferred is defined by the border sequences, and intervening DNA is usually inserted into the plant genome.

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium,* allowing for convenient manipulations as described (Klee et al., In: Plant DNA Infectious Agents, Hohn and Schell, eds., Springer-Verlag, New York, pp. 179-203 (1985). Moreover, technological advances in vectors for *Agrobacterium-mediated* gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate construction of vectors capable of expressing various polypeptide coding genes. The vectors described have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant varieties where *Agrobacterium-mediated* transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

A transgenic plant formed using *Agrobacterium* transformation methods typically contains a single genetic locus on one chromosome. Such transgenic plants can be referred to as being hemizygous for the added gene. More preferred is a transgenic plant that is homozygous for the added structural gene; i.e., a transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants for the gene of interest.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating exogenous

genes. Selfing of appropriate progeny can produce plants that are homozygous for both exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in Fehr, In: Breeding Methods for Cultivar Development, Wilcox J. ed., American Society of Agronomy, Madison Wis. (1987).

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments. Application of these systems to different plant varieties depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described (Fujimura et al., 1985; Toriyama et al., 1986; Abdullah et al., 1986).

Other methods of cell transformation can also be used and include but are not limited to introduction of DNA into plants by direct DNA transfer into pollen, by direct injection of DNA into reproductive organs of a plant, or by direct injection of DNA into the cells of immature embryos followed by the rehydration of desiccated embryos.

The regeneration, development, and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art (Weissbach et al., In: Methods for Plant Molecular Biology, Academic Press, San Diego, Calif., (1988). This regeneration and growth process typically includes the steps of selection of transformed cells; culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil.

The development or regeneration of plants containing the foreign, exogenous gene is well known in the art. Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired exogenous nucleic acid is cultivated using methods well known to one skilled in the art.

Methods for transforming dicots, primarily by use of *Agrobacterium tumefaciens,* and obtaining transgenic plants have been published for cotton (U.S. 5,004,863, U.S. 5,159,135, U.S. 5,518,908); soybean (U.S. 5,569,834, U.S. 5,416,01 1); Brassica (U.S. 5,463,174); peanut (Cheng et al., 1996); and pea (Grant et al., 1995).

Methods for transformation of cereal plants such as wheat and barley for introducing genetic variation into the plant by introduction of an exogenous nucleic acid and for regeneration of plants from protoplasts or immature plant embryos are well known in the art, see for example, CA 2,092,588, AU 61781/94, AU 667939, U.S. 6,100,447, WO 97/048814, U.S. 5,589,617, U.S. 6,541,257, and other methods are set out in WO 99/14314. Preferably, transgenic wheat or barley plants are produced by *Agrobacterium tumefaciens* mediated transformation procedures. Vectors carrying the desired nucleic acid construct may be introduced into regenerable wheat cells of tissue cultured plants or explants, or suitable plant systems such as protoplasts.

The regenerable wheat cells are preferably from the scutellum of immature embryos, mature embryos, callus derived from these, or the meristematic tissue.

To confirm the presence of the transgenes in transgenic cells and plants, a polymerase chain reaction (PCR) amplification or Southern blot analysis can be performed using methods known to those skilled in the art. Expression products of the transgenes can be detected in any of a variety of ways, depending upon the nature of the product, and include Western blot and enzyme assay. One particularly useful way to quantitate protein expression and to detect replication in different plant tissues is to use a reporter gene, such as GUS. Once transgenic plants have been obtained, they may be grown to produce plant tissues or parts having the desired phenotype. The plant tissue or plant parts, may be harvested, and/or the seed collected. The seed may serve as a source for growing additional plants with tissues or parts having the desired characteristics.

In an embodiment, transgenic plants of the invention are produced using methods generally described in US 6,369,299.

Transgenic Non-Human Animals

A "transgenic non-human animal" refers to an animal, other than a human, that contains a gene construct ("transgene") not found in a wild-type animal of the same species or breed. A "transgene" as referred to herein has the normal meaning in the art of biotechnology and includes a genetic sequence which has been produced or altered by recombinant DNA or RNA technology and which has been introduced into an animal cell. The transgene may include genetic sequences derived from an animal cell. Typically, the transgene has been introduced into the animal by human manipulation such as, for example, by transformation but any method can be used as one of skill in the art recognizes.

Techniques for producing transgenic animals are well known in the art. A useful general textbook on this subject is Houdebine, Transgenic animals - Generation and Use (Harwood Academic, 1997).

Heterologous DNA can be introduced, for example, into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal. In a highly preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo. In a most preferred method, however, the appropriate DNAs are coinjected into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals.

Another method used to produce a transgenic animal involves microinjecting a nucleic acid into pro-nuclear stage eggs by standard methods. Injected eggs are then cultured before transfer into the oviducts of pseudopregnant recipients.

Transgenic animals may also be produced by nuclear transfer technology. Using this method, fibroblasts from donor animals are stably transfected with a plasmid incorporating the coding sequences for a binding domain or binding partner of interest under the control of regulatory sequences. Stable transfectants are then fused to enucleated oocytes, cultured and transferred into female recipients.

Compositions

Compositions of the present invention include excipients, also referred to herein as "acceptable carriers". An excipient can be any material that the animal, plant, plant or animal material, or environment (including soil and water samples) to be treated can tolerate. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer, while examples of preservatives include thimerosal or o-cresol, formalin and benzyl alcohol. Excipients can also be used to increase the half-life of a composition, for example, but are not limited to, polymeric controlled release vehicles, biodegradable implants, liposomes, bacteria, viruses, other cells, oils, esters, and glycols.

Furthermore, a polypeptide described herein can be provided in a composition which enhances the rate and/or degree of degradation of a chlorinated s-triazine such as atrazine, or increases the stability of the polypeptide. For example, the polypeptide can be immobilized on a polyurethane matrix (Gordon et al., 1999), or encapsulated in appropriate liposomes (Petrikovics et al., 2000a and b). The polypeptide can also be incorporated into a composition comprising a foam such as those used routinely in fire-fighting (LeJeune et al., 1998). As would be appreciated by the skilled addressee, the polypeptide of the present invention could readily be used in a sponge or foam as disclosed in WO 00/64539.

One embodiment of the present invention is a controlled release formulation that is capable of slowly releasing a composition of the present invention into an animal, plant, animal or plant material, or the environment (including soil and water samples). As used herein, a controlled release formulation comprises a composition of the present invention in a controlled release vehicle. Suitable controlled release vehicles include, but are not limited to, biocompatible polymers, other polymeric matrices, capsules, microcapsules, microparticles, bolus preparations, osmotic pumps, diffusion devices, liposomes, lipospheres, and transdermal delivery systems. Preferred controlled release formulations are biodegradable (i.e., bioerodible).

A preferred controlled release formulation of the present invention is capable of releasing a composition of the present invention into soil or water which is in an area comprising a chlorinated s-triazine. The formulation is preferably released over a period of time ranging from about 1 to about 12 months. A preferred controlled release formulation of the present invention is capable of effecting a treatment preferably for at least about 1 month, more preferably for at least about 3 months, even more preferably for at least about 6 months, even more preferably for at least about 9 months, and even more preferably for at least about 12 months.

The concentration of the polypeptide, vector, or host cell of the present invention that will be required to produce effective compositions for degrading a chlorinated s-triazine, will depend on the nature of the sample to be decontaminated, the concentration of the chlorinated s-triazine in the sample, and the formulation of the composition. The effective concentration of the polypeptide, vector, or host cell within the composition can readily be determined experimentally using a method of the invention.

Enzymes of the invention, and/or host cells encoding therefor, can be used in coating compositions as generally described in WO 2004/112482 and WO 2005/26269.

EXAMPLES

Materials and Methods

Homology modeling

The closest structural relatives of AtzA were identified by submitting the AtzA sequence to the FUGUE server (http://www-crvst.bioc.cam.ac.uk/fugue/) (Shi et al., 2001). The program Swiss-PDBViewer (Kaplan et al., 2001) was used to construct the homology model. The sequence was then manually aligned to build the most reasonable model possible. Particular attention was paid to the alignment of elements of secondary structure, the location of insertions and deletions, the putative substrate-binding pocket and active site residues. This model was then optimized using the web-based SWISS-MODEL server (Schwede et al., 2003), to rebuild side chains and minimize the energy of distorted regions. The positions of seven of the nine amino acid that discriminate TriA and AtzA could be confidently predicted within the model. Images were produced using PyMoI Viewer vθ.99rc6 (DeLano Scientific LLC).

Bacterial growth and DNA manipulation

AtzA was expressed from its native promoter by constructing the pCS150 expression vector, in which the *atzA* promoter from pMD4 was cloned between the *Aval* and *H*mdIII sites of pACYC184 using the primers CS284 (CAACCAATTATCTCGGGGAACTTCTTGAGCGCGGCCACAGCAG) (SEQ ID NO:7) and CS285 (CAACAAGCTTGGATCCTGCAGCTCAGCATGCGGCCGCCATATGATGTCTC CAATAGTGTGTTACAC) (SEQ ID NO:8). The reverse primer (CS285) contained a new multiple cloning site that included an in-frame *Ndel* site and a *BamHl* site that were used to clone the limited site-saturation libraries. AtzA recovered from pCS150 containing wild-type *atzA* had the anticipated specific activity.

Limited site-saturation libraries were constructed by Geneart (http://www.geneart.com/). The quality of the library was such that less than one copy in 20 encoded a mutation in addition to those included by design. DNA sequencing was conducted by Micromon (Department of Microbiology, Monash University, Melbourne, Australia) using the vector-specific primers pCS150For (GACGTGCGGGATGACCACCCAGTTGCGGTGC) (SEQ ID NO:9) and pCS 150Rev (GAGATTACGAGAAGACCAAAACGATCTCAAGAAGATCATC) (SEQ ID NO: 10), and the *atzA*-specific primer *atzA529F* (CAAGTCGAACTGTGCTCGAT) (SEQ ID NO: 11). The pCS150 derivates containing the libraries were used to transform chemically competent *E. coli* JM 109

(Promega). *E. coli* were grown on LB (Lennox, 1955) supplemented with 15 gl^{-1} agar, 34 $\mu g ml^{-1}$ chloramphenicol and 1 $mg ml^{-1}$ atrazine as required.

Variant enzymes with potentially improved atrazine dechlorinase activity were selected from the library on the basis of their capacity to confer an enhanced capacity to produce zones of clearance on atrazine-containing agar upon the host JM109 colony when compared with JMl 09 expressing wild-type *atzA.* Colonies expressing wild-type AtzA enzyme produced visible zones of clearance after 48 hours growth at 37 ^{0}C.

Protein preparation and enzyme kinetics

AtzA variants were expressed from pCS150, grown in 2 1 shake flasks (shaken at 250 rpm) in LB supplemented with chloramphenicol at 37 C for 48 hours. Cultures were sedimented by centrifugation and suspended in AtzA reaction buffer (de Souza et al., 1996). Cells were lysed by freeze/thaw, followed by sonication (Brannson Sonifier 250). Lysates were then clarified by centrifugation. AtzA and its variants were then enriched to greater than 50% of the total protein by the ammonium-sulphate precipitation method described by de Souza et al. (1996), and quantified by denaturing PAGE gel densitometry. Samples of bovine serum albumin (BSA; 5, 10 and 20 μg) were loaded onto each gel analysed by densitometry, to provide an internal standard.

Specific activities were obtained for every variant that conferred a clearing phenotype to JM109 cells that was superior to that of wild-type AtzA using the method of Tawfik et al. (1968), adapted to a 96-well microtitre plate format. The specific activity assays were performed at 23 μM atrazine (equivalent to approximately 16 % of the published K_M of AtzA for atrazine (de Souza et al., 1996). Kinetic analyses of the wild-type AtzA, the ten variants from the limited site-saturation library with the highest specific activities and the AtzA containing the consensus *N*-ethyl pocket were determined by the same method, using atrazine concentrations between 4.6 and 153 μM. Where possible the K_M and V_{max} for each reaction was calculated using a curve fitting program (Curve Expert 1.3; http://curveexpert.webhop.biz/).

Results and Discussion

Construction of structural model

AtzA is known to be a metallohydrolase from the urease/amidohydrolase superfamily (de Souza et al., 1996), although the atomic structure is yet to be elucidated. To construct a homology model of AtzA, its closest structural homologues were identified and used as templates. The empirically determined

structures of a protein of unknown function from *Thermotoga maritima* (lj óp; unpublished database entry), the imidazolone propionase from *Agrobacterium tumefaciens* (2gok; unpublished database entry), and the *Escherichia coli* cytosine deaminase (Ik6w) (Ireton et al., 2002) were identified using the FUGUE server as being the most closely related to that of AtzA. As the structures and the substrates of these enzymes are similar to atrazine (Figure 1), it was considered that they were suitable candidates for modelling the AtzA:atrazine complex.

Atrazine was docked into the active site of the AtzA model by superimposing the molecule onto the structure of the imidazolone propionase in complex with 4-imidazolone-5-propanoate. On this basis, the residues that form the active site and substrate binding pocket were identified (Figure 2). Three regions were not altered: residues H66, H68, H243, E246, H276 and D327, which are essential for coordination of the active-site metal ion (Seibert and Raushel, 2005); residues N328 and S331, that have been shown to be essential in the atrazine dechlorinase activity (Raillard et al., 2001); residues F84, W87 and L88 that form the hydrophobic "base" of the active site, and are likely to be essential for π-π interaction with the aromatic ring of the substrate. The remaining amino acid resides are proposed to interact with either the *N*-ethyl (A216, T217, T219, A220 and D250) or *N*-isopropyl side-chain groups of atrazine (Q71, L88, V92, Q96, E125, N126, D128, Ml 55 andN330).

Limited site-saturation mutagenesis

The model was used to predict amino acid targets in the active site that may influence the binding affinity of the substrate. Examination of the *N*-ethyl and N-isopropyl side chain-binding "pocket" in the AtzA:atrazine model suggested that the K_M of AtzA for atrazine could be reduced somewhat by altering the composition of this group of amino acids to better accommodate and/or make more productive interactions with the substrate. Two limited site saturation (LSS) libraries were constructed (GeneART); one for the *N*-ethyl side chain-binding pocket, and one for the *N*-isopropyl side chain-binding pocket. In the N-ethyl binding pocket LSS library the codons for five amino acid residues (A216, T217, T219, A220 and D250) from the *N*-ethyl binding pocket replaced with the degenerate codon BNS (where B is T, G or C, N is A, T, G or C and S is C or G; Table 2). In the N-isopropyl binding pocket LSS library the codons for five amino acid residues (Q71, L88, V92, Q96 and N330) from the *N*-isopropyl binding pocket replaced with BNS.

The BNS degeneracy allows screening of fifteen of the twenty possible amino acids (methionine, isoleucine, threonine, asparagine and lysine are not encoded by any combinations these nucleotides); largely maintaining the biochemical diversity of the library, whilst reducing the diversity of the library at the nucleotide level *(ca.* 8×10^6

combinations with 5 degenerate codons, rather than 1 x 10^9 generated by the equivalent 64 codon library). Two of the three stop codons were avoided by using the BNS degeneracy, and the discrepancy in the frequencies of the most and least highly represented amino acids was reduced from 6:1 to 3:1. The variant enzymes encoded by these libraries were screened by clearing plate assay for their ability to dechlorinate atrazine. From approximately 10^6 clones from each library 298 TV-ethyl and 58 TV-isopropyl binding-pocket variants were selected and their specific activities determined. 50 % of the TV-ethyl pocket variants and 20 % of the TV-isopropyl pocket variants had greater than 2-fold higher specific activity compared with the wild-type (Table 3).

Table 2. Amino acids encoded by the BNS degeneracy.

1st base (B)	2nd base (N)				3rd base (S)
	T	C	A	G	
T	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Stop	Trp	G
C	Leu	Pro	His	Arg	C
	Leu	Pro	GIn	Arg	G
G	VaI	Ala	Asp	GIy	C
	VaI	Ala	GIu	GIy	G

Table 3. Distribution of specific activity enhancements in the iV-ethyl and TV-isopropyl limited site saturation libraries. Specific activities were determined using a 23 μM initial concentration of atrazine and 34.6 nM enzyme.

Fold enhancement (specific activity)	TV-ethyl binding pocket library	TV-isopropyl binding pocket library
> 2	50.0	80.0
2 - 3	6.8	6.7
3 - 4	10.4	0.0
4 - 5	7.9	2.2
5 - 10	17.7	8.9
10 - 15	6.5	2.2
15 - 20	0.3	0.0
> 20	0.3	0.0

Selection from the /V-isopropyl library yielded far fewer colonies with improved clearing than the TV-ethyl library (58 TV-isopropyl compared with 298 TV-ethyl). This is at least in part to the stringent amino acid requirements at residues 88 and 330 (Table 4). Sixty percent of the TV-isopropyl pocket library that allowed

improved clearing possessed the wild-type leucine at residue 88, suggesting that it is unlikely it can be substituted by another amino acid effectively.

Table 4. Amino acids selection and counter-selection in the enriched *N*-isopropyl-binding pocket libraries. Values indicate the frequency of each amino acid at each position as a percentage of the total. The values in parentheses indicate fold-differences between the frequencies of each amino acid and that which would be expected by random chance alone (based on codon frequency).

	Randomised residue				
Amino acid	**71**	**88**	**92**	**96**	**330**
A	11 (1.3)	10 (1.2)	35 (4.2)	26 (3.2)	15 (1.8)
L	28 (2.2)	60 (4.8)	5 (0.4)	-	-
G	11 (1.3)	10 (1.2)	20 (2.4)	16 (1.9)	20 (2.4)
V	-	10 (1.2)	-	10 (1.2)	-
S	6 (0.7)	-	-	21 (2.5)	5 (0.6)
Q	11 (2.7)	-	5 (1.2)	5 (1.2)	5 (1.2)
Y	6 (1.3)	-	-	-	-
P	28 (3.3)	-	5 (0.6)	5 (0.6)	-
D	-	-	-	-	-
E	-	-	-	-	5 (1.2)
R	-	-	-	5 (0.6)	10 (1.2)
C	-	5 (1.2)	5 (1.2)	-	35 (8.4)
H	-	-	10 (2.4)	-	5 (1.2)
W	-	5 (1.2)	15 (3.6)	-	-
F	-	-	-	11 (2.5)	-
Wild-type	**Q**	**L**	**V**	**Q**	**N**
Selected*	PLQ	L	AWHG	ASF	CG
Counter selected†	FWHCRE DV	FHR EDP QS	FRE DYS VL	WHC EYDL L	FWP YVL

* Amino acid occurs more than twice as often as expected by random chance alone.

† Amino acid occurs less than half as often as expected by random chance alone.

At residue 330 cysteine, glycine and alanine represented 70% of the diversity in the selected variant enzymes, with the cysteine specific codon being selected more than eight times more frequently than expected by random chance would allow (Table

4). At this position it was impossible to reconstitute the wild-type sequence as asparagine was not encoded by any combination of the BNS degeneracy, and it appears that asparagine can be most successfully substituted with a small uncharged amino acid residue at this position.

Surprisingly, the wild-type amino acid residue at position 92 (valine) is extremely underrepresented in the variants selected. V92 is one of the nine amino acid residues that vary between AtzA and TriA, and may have a role in determining the substrate specificity of the enzyme; its absence in the variants selected here is therefore seemingly paradoxical. In the modeled structure V92 is situated in close proximity to N330 (Figure 2), and it may be the interaction between N330 and V92 that has been optimised during natural evolution, rather than the presence of V92 itself. So by eliminating asparagine from residue 330, the amino acid preference at residue 92 was also changed. There was an apparent selection against large and charged amino acids at position 71; leucine and proline being most abundant, followed by alanine and the wild-type residue (glutamine). Alanine and serine were the amino acids most frequently found at position 96, rather than the wild-type (glutamine).

The iV-ethyl library appeared to be able to tolerate reasonably high levels of diversity without leading to a loss of activity, yielding nearly 300 variants with greater apparent activity than wild-type AtzA as judged by plate clearing assay.

Variation within the pockets may be affecting factors other activity *per se* (e.g. expression levels or solubility). To identify variants within the selected samples that did not impact activity directly, specific activity data were obtained for each of the variants that conferred and enhanced clearing phenotype upon the *E. coli* expression host. The specific activities of the variant enzymes enriched from the LSS libraries were determined at a substrate concentration of 23 μM (a sixth of the published value for the K_M of AtzA, 149 μM (de Souza et al., 1996), and compared with the wild-type AtzA. Half of the *N*-ethyl pocket had specific activities at least twice that of the wild-type enzyme under these conditions (Table 3). The *N*-isopropyl library contained only five variants with greater than five-fold the specific activity of the wild-type enzyme (Table 3), three of which had identical amino acid sequences (L71, L88, G92, A96, C330).

A consensus of the amino acid preferences in the *N*-ethyl variants with five¬ fold or greater improvements in their specific activities was produced, to determine a consensus specific for variants with increased specific activities at lower substrate concentration (presumably due to improved binding affinities) (Table 5 for consensus). The consensus variant (A216Y, T217D, A220H, D250E) had not been selected from the library. The consensus variant was generated by directed

mutagenesis and a fast clearing phenotype upon *E. coli* JM109, and was therefore further examined in more complete kinetic analysis.

Table 5. Amino acid selection and counter-selection in the *N*-ethyl binding pocket variants with five-fold or greater improvements in specific activity when compared to wild-type AtzA. Assays done at 23 μM atrazine and 34.6 nM enzyme. Values indicate the frequency of each amino acid at each position as a percentage of the total. The values in parentheses indicate fold-differences between the frequencies of each amino acid and that which would be expected by random chance alone (based on codon frequency).

	Randomised residue									
Amino Acid	**216**		**217**		**219**		220		250	
A	26.1	(3.1)	21.7	(2.6)	4.3	(0-5)	26.1	(3.1)	4.3	(0.5)
L	-		-		8.7	(0.7)	-		4.3	(0.3)
G	13.0	(1.6)	26.1	(3.1)	21.7	(2.6)	4.3	(0.5)	17.4	(2.1)
V	-		-		8.7	(1.0)	4.3	(0.5)	4.3	(0.5)
S	17.4	(2.1)	8.7	(1.0)	4.3	(0.5)	26.1	(3.1)	8.7	(1.0)
Q	-		-		-		4.3	(1.0)	-	
Y	34.8	(8.3)	-		8.7	(2.1)	-		8.7	(2.1)
P	-		-		13.0	**(1.5)**	4.3	(0.5)	4.3	(0.5)
D	-		43.5	(10.4)	13.0	(1.5)	-		8.7	(2.1)
E	-		-		8.7	(2.1)	-		21.7	(5.2)
R	-		-		4.3	(0.5)	-		-	
C	-		-		-		-		4.3	(1.0)
H	8.7	(2.1)	-		4.3	(1.0)	21.7	(5.2)	4.3	(1.0)
W	-		-		-		4.3	(1.0)	8.7	(2.1)
F	-		-		-		4.3	(1.0)	-	
Consensus	YAS		DGA		GYE		HAS		EDGYW	
Wild-type	A		T		T		T		D	

* Amino acid occurs more than twice as often as expected by random chance alone.
† Amino acid occurs less than half as often as expected by random chance alone.

Wild-type AtzA, five most improved N-isopropyl, ten most improved N-ethyl and the consensus *N*-ethyl binding pocket variant were then assayed to obtain their kinetic data (Tables 6 and 7 for N-ethyl, and Tables 8 and 9 for N-isopropyl, data). The relationship between the reaction rate of the wild-type AtzA enzyme and

substrate concentration was linear up to the aqueous solubility limit of atrazine (153 μM; The Pesticide Manual), this was confirmed by LC-MS analysis of hydroxyatrazine production. Thus, it was not possible to corroborate the previous determination of K_M *of* AtzA (149 μM) (de Souza et al., 1996). Surprisingly, the data presented here suggest that the K_M of AtzA for atrazine significantly exceeds the atrazine's water solubility (Figure 3). Surprisingly, unlike wild-type AtzA, the improved variants displayed a sigmoidal response (with r-values of 0.996-0.999; Figure 3). This observation was confirmed by LC-MS analysis of hydroxyatrazine accumulation. Such a relationship suggests that AtzA may be subject to substrate-dependent cooperative behavior.

Table 6. Characterisation of the most improved AtzA variants from the *N*-ethyl pocket limited site-saturation mutagenesis library.

Clone	Amino acid at position;					*KM*	k_{cat}	Specific activity
	216	217	219	220	250	(μM)	(s^{-1})	(s^{-1})*
AtzA	-	-	-	-	-	>153*	ND‡	0.07
;422	Y	D	Y	H	V	92	6.5	0.72
;357	S	A	P	F	G	67	2.3	0.76
e430	G	D	G	H	G	90	12.7	0.76
E431	A	S	H	G	Y	76	8.0	0.77
e297	S	D	V	H	G	92	6.8	0.77
L-288	A	D	E	A	D	49	4.3	0.80
e662	H	A	E	S	S	100	7.4	1.05
e305	G	D	A	V	W	95	6.2	1.08
e841	S	D	G	S	D	105	8.5	1.12
e734	G	D	G	H	D	62	15.1	1.45
consensus	Y	D	T	H	E	93	27.9	ND

ND Not determined.

* Specific activity determined with an initial concentration of 23 μM atrazine.

† Theoretic value calculated by

‡ As the K_M of AtzA for atrazine greatly exceeded the water solubility of atrazine it was not possible to determine these values.

Table 7. Amino acids at randomised positions (216, 217, 219, 220 and 250) of *N*-ethyl pocket variants with better than 2-fold enhanced activity compared with wild-type AtzA. The sequences of variants with 5-fold or greater activity than wild-type have been boldfaced, as these are the preferred sequences that were isolated, and were used to derive the consensus (Table 5). The 10 most active variants (boldface and italic) were used to obtain kinetics to confirm the specific activity data (Figure 3 and Table 6).

Amino Acid position:					**Relative**	**Specific**
216	**217**	**219**	**220**	**250**	**Specific**	Activity
					Activity*	**(niM.s¯'.mM)**
G	**G**	**V**	**H**	**D**	2.2	0.16
Y	**G**	**A**	**P**	**R**	2.2	0.16
G	**S**	**E**	**Y**	**P**	**2.3**	0.17
Y	**A**	**G**	**P**	C	**2.5**	0.18
Y	**A**	**P**	**Y**	**A**	**2.5**	0.18
A	**A**	**A**	**L**	**H**	**3.2**	0.23
Y	**A**	**Q**	**F**	**S**	**3.3**	0.24
W	**D**	**G**	**H**	**R**	**3.6**	0.26
S	**A**	**A**	**S**	**S**	**3.6**	0.26
A	**Q**	**A**	C	**F**	**3.7**	0.27
G	**S**	**D**	**D**	**D**	**3.7**	0.27
A	**S**	**Y**	**L**	**R**	**4.0**	0.28
H	**G**	**G**	**Y**	**V**	**4.0**	0.28
A	**S**	**Q**	**Q**	**G**	4.1	0.29
H	C	**G**	**G**	**E**	**4.3**	0.31
L	**D**	**H**	**Q**	**V**	**4.5**	0.33
R	**A**	**S**	**R**	**A**	**5.0**	0.36
Y	**A**	**G**	**P**	**C**	**5.8**	**0.42**
Y	**G**	**D**	**S**	**E**	**6.4**	**0.46**
A	**G**	**D**	**A**	**A**	**6.9**	**0.50**
S	**G**	**R**	**S**	**E**	**6.9**	**0.50**
Y	**D**	**V**	**A**	**G**	**7.1**	**0.51**
Y	**G**	**L**	**S**	**L**	**7.3**	**0.53**
A	**D**	**S**	**W**	**S**	**7.7**	**0.56**
A	**G**	**P**	**A**	**W**	**7.9**	**0.57**
Y	**A**	**G**	**A**	**Y**	**7.9**	**0.57**
Y	**S**	**P**	**S**	**P**	**8.6**	**0.62**

Y	G	Y	A	**H**	8.7	**0.63**
H	A	L	H	E	**8.8**	**0.63**
G	D	G	H	E	9.2	**0.66**
Y	D	Y	H	V	**10.0**	**0.72**
S	A	P	F	G	**10.4**	**0.76**
G	D	G	H	G	**10.5**	**0.76**
A	S	H	G	Y	**10.7**	**0.77**
S	D	V	H	G	**10.7**	**0.77**
A	D	E	A	D	**11.1**	**0.80**
H	A	E	S	S	**14.5**	**1.05**
G	D	A	V	W	**15.0**	**1.08**
S	D	G	S	D	**15.5**	**1.12**
G	**D**	G	H	D	**20.1**	**1.45**

* Compared with wild-type with 23 μM atrazine and 37.5 nM enzyme.

All ten of the improved variants had improved binding affinities *(KM,* assuming that AtzA exhibits atrazine-dependent cooperativity) when compared with the wild-type, with the lowest K_M being 49 μM (*N*-ethyl variant 288, Table 6). However, as the K_M of wild-type AtzA could not be determined it is impossible to quantify to extent of this enhancement. The maximum K_{cat} from a variant isolated from the LSS library was approximately 15 sec^{-1}, observed with the *N*-ethyl variant 734 (Table 6). There was also an increase in the K_{cat} of the most active *N*-isopropyl pocket variant compared with the wild-type enzyme (5.2 s^{-1} for variants 4, 6 and 7, all of which were identical in sequence) (Table 8). Again the selection for a reduced K_n, was successful, with all of the tested variants exhibiting Km values of less than half the water solubility of the substrate (Table 8), the lowest K_m at 70 μM for the most active variant (4, 6 and 7).

On the basis of its enhanced clearing phenotype, kinetic data were obtained for the "consensus" *N*-ethyl pocket variant. The K_{cat} of this variant exceeded that of the best variant selected form the library (approximately 22 sec^{-1}), with a K_M of 84 μM. Based on the rate of spontaneous, non-catalysed dechlorination of atrazine (k_{non}) estimated by Lei and coworkers (2001), the rate enhancement (k_{ca}/k_{non}) of the consensus *N*-ethyl pocket variant generated in this study was equivalent to approximately 1.3×10^8.

Table 8. Characterisation of the most improved AtzA variants from the *N*-isopropyl pocket limited site-saturation mutagenesis library.

Clone	Amino acid at position;					K_M (μM)	k_{cat} (s^{-1})	Specific activity (s^{-1})*
	71	88	92	*96*	330			
AtzA	**Q**	**L**	**V**	**Q**	**N**	>153*	ND*	0.07
5	G	L	W	V	C	73	1.5	0.46
9	A	L	A	A	C	75	2.6	0.44
4	L	L	G	A	C	70	5.2	0.73
6	L	L	G	A	C	70	5.2	0.73
7	L	L	G	A	C	70	5.2	0.73

Table 9. Amino acids at randomised positions (71, 88, 92, 96 and 330) of W-ethyl pocket variants with better than 2-fold enhanced activity compared with wild-type AtzA. The most active variants (boldface and italic) were used to obtain kinetics to confirm the specific activity data (Figure 3 and Table 8).

Amino Acid position:					**Relative**	Specific
71	**88**	**92**	**96**	**330**	**Specific**	Activity
					Activity*	($mM.s^{-1}$.mM)
G	L	A	A	C	2.0	0.14
G	L	A	S	C	2.0	0.14
A	L	G	S ..	C	2.1	0.15
L	W	A	F	C	2.4	0.18
A	L	W	F	C	2.7	0.19
G	L	H	S	C	4.4	0.32
A	*L*	*A*	*A*	**C**	*6.1*	*0.44*
G	*L*	*W*	*V*	**C**	*6.4*	*0.46*
L	*L*	*G*	*A*	*C*	*10.1*	*0.73*
L	*L*	*G*	*A*	*C*	*10.1*	*0.73*
L	*L*	*G*	*A*	*C*	*10.1*	*0.73*

* Compared with wild-type with 23 μM atrazine and 37.5 nM enzyme.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

The present application claims priority from US 61/014,905 filed 19 December 2007, the entire contents of which are incorporated herein by reference.

All publications discussed and/or referenced herein are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

REFERENCES

Abdullah et al. (1986) Biotechnology 4:1087.
Alcalde et al. (2007) Biocatal. Biotransform. 25:1 13-1 13.
Belluck et al. (1991) Acs Symposium Series 459:254-273.
Boundy-Mills et al. (1997) Appl. Environ. Microbiol. 63:916-923.
Capecchi (1980) Cell 22:479-488.
Cheng et al. (1996) Plant Cell Rep. 15:653-657.
Cheng et al. (2005) Appl. Environ. Microbiol. 71:4437-4445.
Clapp (1993) Clin. Perinatol. 20:155-168.
Curiel et al. (1992) Hum. Gen. Ther. 3:147-154.
de Souza et al. (1995) Appl. Environ. Microbiol. 61:3373-3378.
de Souza et al. (1996) J. Bacteriol. 178:4894-4900.
Eglitis et al. (1988) Biotechniques 6:608-614.
Fruchey et al. (2003) Appl. Environ. Microbiol. 69:3653-3657.
Fujimura et al. (1985) Plant Tissue Culture Letters 2:74.
Gavrilescu (2005) Eng. Life Sci. 5, 497-526.
Gordon et al. (1999). Chemical-Biological Interactions 14:463-470.
Graham et al. (1973) Virology 54:536-539.
Grant et al. (1995) Plant Cell Rep. 15:254-258.
Harayama (1998) Trends Biotechnol. 16:76-82.
Hayes et al. (2002) Proc. Natl. Acad. Sci. U. S. A. 99:5476-5480.
Hayes et al. (2003) Environ. Health Perspect. 111:568-575.
Hayes et al. (2006) Environ. Health Perspect. 114:134-141.
Huff (2002) Int. J. Occup. Environ. Health 8:249-270.
Huff and Sass, J. (2007) Int. J. Occup. Environ. Health 13, 356-358.
Ireton et al. (2002) J. MoI. Biol. 315:687-697.
Kaplan et al. (2001) Brief Bioinform. 2:195-197.
Koziel et al. (1996) Plant MoI Biol 32:393-405.
Lei et al. (2001) J. Environ. Sci. (China) 13:99-103.
LeJuene et al. (1998). Nature 395:27-28.
Lennox (1955) Virol. 1:190-206.
Lu et al. (1993) J. Exp. Med. 178:2089-2096.
Mandelbaum et al. (1995) Appl. Environ. Microbiol. 61:1451-1457.
Martinez et al. (2001) J. Bacteriol. 183:5684-5697.
Needleman and Wunsch (1970), J. MoI. Biol., 48:443-453.
Parales et al. (2002) Appl. Environ. Microbiol. 68:4699-4709.
Petrikovics et al. (2000a) Toxicology Science 57: 16-21.

Petrikovics et al. (2000b) Drug Delivery 7: 83-89.
Raillard et al. (2001) Chem. Biol. 8:891-898.
Sadowsky et al. (1998) J. Bacteriol. 180:152-158.
Schwede et al. (2003) Nucleic Acids Research 31:3381-3385.
Seffernick and Wackett (2001) Biochemistry 40:12747-12753.
Seffernick et al. (2001) J. Bacteriol. 183:2405-2410.
Seibert and Raushel (2005) Biochemistry 44: 6383-6391.
Shapir et al. (2005) J. Bacteriol. 187:3731-3738.
Shi et al. (2001) J. MoI. Biol. 310:243-257.
Sutherland et al. (2004) Clin. Exp. Pharmacol. Physiol. 31:817-821.
Tawfik et al. (1968) J. Agr. Food Chem. 16:284-289.
Thurman and Meyer (1996) Herbicide metabolites in surface water and groundwater: Introduction and overview, in Herbicide Metabolites in Surface Water and Groundwater
Tomlin (2006) The Pesticide Manual BCPB.
Toriyama et al. (1986) Theor. Appl. Genet. 205:34.
van der Meer (2006) Front. Ecol. Environ. 4:35-42.
Wagner et al. (1992) Proc. Natl. Acad. Sci. USA 89:6099-6103.

CLAIMS

1. A substantially purified and/or recombinant polypeptide which has a KM for atrazine which is less than 150μM.

2. The polypeptide of claim 1 which has a K_M for atrazine which is less than 100μM.

3. A substantially purified and/or recombinant polypeptide which has at least a two-fold greater specific activity against atrazine than a polypeptide with a sequence provided as SEQ ID NO: 1.

4. The polypeptide of claim 3 which has at least a five-fold greater specific activity against atrazine than the polypeptide with a sequence provided as SEQ ID NO:1.

5. A substantially purified and/or recombinant polypeptide comprising amino acids having a sequence which is at least 80% identical to any one or more of SEQ ID NO's 1 to 3, wherein the polypeptide comprises

i) a tyrosine, alanine, serine, glycine, tryptophan, leucine, arginine or histidine at a position corresponding to amino acid number 216, of SEQ ID NO:1, and/or

ii) an alanine, serine, glycine, glutamine, cysteine or aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, and/or

iii) a tyrosine, alanine, serine, glycine, aspartic acid, arginine, valine, leucine, proline, histidine, glutamine or glutamic acid at a position corresponding to amino acid number 219 of SEQ ID NO: 1, and/or

iv) an alanine, serine, glycine, valine, proline, histidine, tryptophan, phenylalanine, tyrosine, leucine, cysteine, aspartic acid, arginine or glutamine at a position corresponding to amino acid number 220 of SEQ ID NO:1, and/or

v) a serine, glycine, valine, proline, histidine, tryptophan, cysteine, glutamic acid, alanine, leucine, tyrosine, arginine, phenylalanine or aspartic acid at a position corresponding to amino acid number 250 of SEQ ID NO:1, and/or

vi) a biologically active fragment of any one of i) to v),

and wherein the polypeptide degrades a chlorinated s-triazine, and wherein if alanine is present at position 216 the polypeptide has at least one of the other amino acid listed in ii) to v), and wherein if alanine is present at position 220 the polypeptide has at least one of the other amino acid listed in i) to iii) or v), and wherein if aspartic acid

is present at position 250 the polypeptide has at least one of the other amino acid listed in i) to iv).

6. A substantially purified and/or recombinant polypeptide comprising amino acids having a sequence which is at least 80% identical to any one or more of SEQ ID NO's 1 to 3, wherein the polypeptide comprises

i) an alanine, glycine or leucine at a position corresponding to amino acid number 71 of SEQ ID NO:1, and/or

ii) a leucine or tryptophan at a position corresponding to amino acid number 88 of SEQ ID NO: 1, and/or

iii) an alanine, glycine, histidine or tryptophan at a position corresponding to amino acid number 92 of SEQ ID NO: 1, and/or

iv) an alanine, serine, phenylalanine or valine at a position corresponding to amino acid number 96 of SEQ ID NO: 1, and/or

v) a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:l, or

vi) a biologically active fragment of any one of i) to iv),

and wherein the polypeptide degrades a chlorinated s-triazine, and wherein if leucine is present at position 88 the polypeptide has at least one of the other amino acid listed in i) or iii) to v).

7. The polypeptide of claim 5 or claim 6, wherein the chlorinated s-triazine is atrazine, and the polypeptide has at least a two-fold greater specific activity against atrazine than the polypeptide provided as SEQ ID NO: 1.

8. A substantially purified and/or recombinant polypeptide comprising amino acids having a sequence which is at least 80% identical to any one or more of SEQ ID NO's 1 to 3, wherein the polypeptide comprises

i) a tyrosine, alanine, serine, glycine or histidine at a position corresponding to amino acid number 216 of SEQ ID NO:1, and/or

ii) an alanine, serine, glycine or aspartic acid at a position corresponding to amino acid number 217 of SEQ ID NO:1, and/or

iii) a tyrosine, alanine, serine, glycine, aspartic acid, arginine, valine, leucine, proline, histidine or glutamic acid at a position corresponding to amino acid number 219 of SEQ ID NO: 1, and/or

iv) an alanine, serine, glycine, valine, proline, histidine, tryptophan or phenylalanine at a position corresponding to amino acid number 220 of SEQ ID NO: 1, and/or

v) a serine, glycine, valine, proline, histidine, tryptophan, cysteine, glutamic acid, alanine, leucine, tyrosine or aspartic acid at a position corresponding to amino acid number 250 of SEQ ID NO: 1, and/or

vi) a biologically active fragment of any one of i) to v),

and wherein the polypeptide degrades a chlorinated s-triazine, and wherein if alanine is present at position 216 the polypeptide has at least one of the other amino acids listed in ii) to v), and wherein if alanine is present at position 220 the polypeptide has at least one of the other amino acid listed in i) to iii) or v), and wherein if aspartic acid is present at position 250 the polypeptide has at least one of the other amino acids listed in i) to iv).

9. A substantially purified and/or recombinant polypeptide comprising amino acids having a sequence which is at least 80% identical to any one or more of SEQ ID NO' s 1 to 3, wherein the polypeptide comprises

i) an alanine, glycine or leucine at a position corresponding to amino acid number 7 1 of SEQ ID NO:1, and/or

ii) an alanine, glycine or tryptophan at a position corresponding to amino acid number 92 of SEQ ID NO: 1, and/or

iii) an alanine or valine at a position corresponding to amino acid number 96 of SEQ ID NO: 1, and/or

iv) a cysteine at a position corresponding to amino acid number 330 of SEQ ID NO:l, or

v) a biologically active fragment of any one of i) to iv),

and wherein the polypeptide degrades a chlorinated s-triazine.

10. The polypeptide of claim 8 or claim 9, wherein the chlorinated s-triazine is atrazine, and the polypeptide has at least a five-fold greater specific activity against atrazine than the polypeptide provided as SEQ ID NO: 1.

11. The polypeptide according to any one of claims 5, 6, 8 or 9, wherein the chlorinated s-triazine is atrazine or simazine.

12. The polypeptide according to any one of claims 1 to 11, which is a fusion protein further comprising at least one other polypeptide sequence.

13. An isolated and/or exogenous polynucleotide encoding a polypeptide according to any one of claims 1 to 12.

14. The polynucleotide of claim 13 which comprises a sequence which is at least 80% identical to any one or more of SEQ ID NO's 4 to 6.

15. The polynucleotide of claim 13 or claim 14 which is operably linked to a promoter capable of directing expression of the polynucleotide in a cell.

16. A vector comprising a polynucleotide according to any one of claims 13 to 15.

17. A host cell comprising a polynucleotide according to any one of claims 13 to 15 and/or a vector of claim 16.

18. The host cell of claim 17 which is a bacterial cell or a yeast cell.

19. A transgenic plant comprising at least one cell of claim 18.

20. A transgenic non-human animal comprising at least one cell of claim 18.

21. An extract of a host cell of claim 17 or claim 18, the plant of claim 19 and/or the animal of claim 20, wherein the extract comprises a polypeptide according to any one of claims 1 to 12.

22. A composition comprising a polypeptide according to any one of claims 1 to 12, a polynucleotide according to any one of claims 13 to 15, a vector of claim 16, a host cell of claim 17 or claim 18 and/or extract of claim 21, and one or more acceptable carriers.

23. A method for degrading a chlorinated s-triazine, the method comprising contacting the chlorinated s-triazine with a polypeptide according to any one of claims 1 to 12, a polynucleotide according to any one of claims 13 to 15, a vector of claim 16, a host cell of claim 17 or claim 18, a plant of claim 19, an animal of claim 20, an extract of claim 21, and/or a composition of claim 22.

24. The method of claim 23, wherein the chlorinated s-triazine is in a sample selected from the group consisting of: soil; water, biological material or a combination thereof.

25. A polymeric sponge or foam for degrading a chlorinated s-triazine, the foam or sponge comprising a polypeptide according to any one of claims 1 to 12 immobilized on a polymeric porous support.

26. A method for degrading a chlorinated s-triazine, the method comprising contacting the chlorinated s-triazine with a sponge or foam of claim 25.

27. A method for degrading a chlorinated s-triazine, the method comprising,

i) determining the concentration of the chlorinated s-triazine,

ii) determining the area comprising the chlorinated s-triazine,

iii) calculating the concentration of polypeptide required to degrade the chlorinated s-triazine using a sigmoidal profile,

iv) contacting the chlorinated s-triazine with the polypeptide.

28. The method of claim 27, wherein the polypeptide is a polypeptide according to any one of claims 1 to 12.

29. The method of claim 27 or claim 28, wherein the chlorinated s-triazine is atrazine.

30. A method of treating toxicity caused by an chlorinated s-triazine in a subject, the method comprising administering to the subject a polypeptide according to any one of claims 1 to 12, a polynucleotide according to any one of claims 13 to 15, a vector of claim 16, a host cell of claim 17 or claim 18, a plant of claim 19, an animal of claim 20, an extract of claim 21 and/or a composition of claim 22.

31. Use of a polypeptide according to any one of claims 1 to 12, a polynucleotide according to any one of claims 13 to 15, a vector of claim 16, a host cell of claim 17 or claim 18, a plant of claim 19, an animal of claim 20, an extract of claim 21 and/or a composition of claim 22 for the manufacture of a medicament for treating toxicity caused by chlorinated s-triazine in a subject.

32. A method of producing the polypeptide according to any one of claims 1 to 12, the method comprising expressing in a cell, or a cell-free expression system, the polynucleotide according to any one of claims 13 to 15 and/or a vector of claim 16.

33. A method for detecting a host cell, the method comprising

i) contacting a cell or a population of cells with a polynucleotide according to any one of claims 13 to 15 under conditions which allow uptake of the polynucleotide by the cell(s), and

ii) selecting a host cell by exposing the cells from step i), or progeny cells thereof, to a chlorinated s-triazine.

34. The method of claim 33, wherein the polynucleotide comprises a first open reading frame comprising a polynucleotide according to any one of claims 13 to 15, and a second open reading frame not comprising a polynucleotide according to any one of claims 13 to 15.

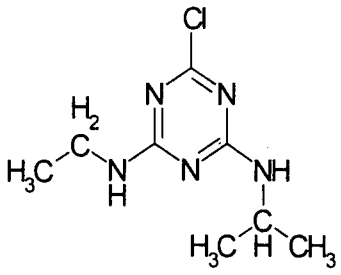
35. The method of claim 34, wherein the second open reading frame encodes a polypeptide.

36. The method of claim 34, wherein the second open reading frame encodes a polynucleotide which is not translated.

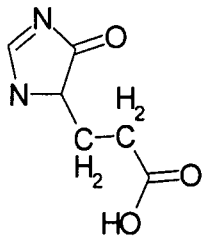
37. The method of claim 36, wherein the polynucleotide which is not translated encodes a catalytic nucleic acid, a dsRNA molecule, or an antisense molecule.

38. The method according to any one of claims 33 to 37, wherein the cell is a plant cell.

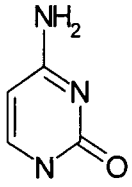
39. A kit for degrading a chlorinated s-triazine, the kit comprising a polypeptide according to any one of claims 1 to 12, a polynucleotide according to any one of claims 13 to 15, a vector of claim 16, a host cell of claim 17 or claim 18, a plant of claim 19, an animal of claim 20, an extract of claim 21, a composition of claim 22 and/or a sponge or foam of claim 25.



Atrazine



4-Imidazolone-5-propanoate



Cytosine

Figure 1

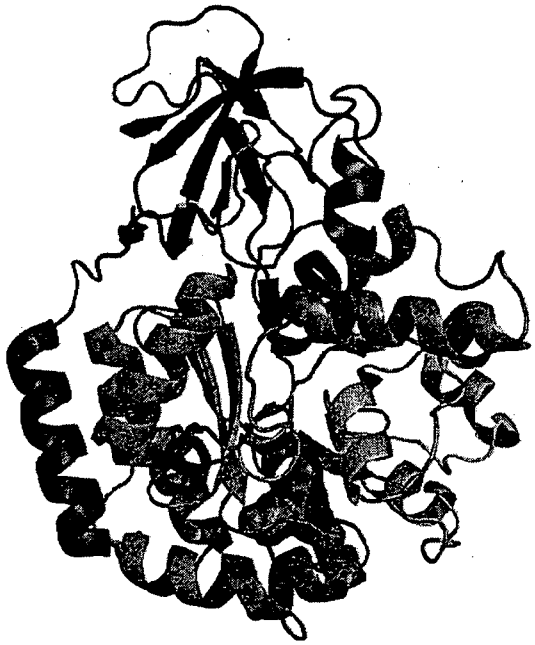


Figure 2a

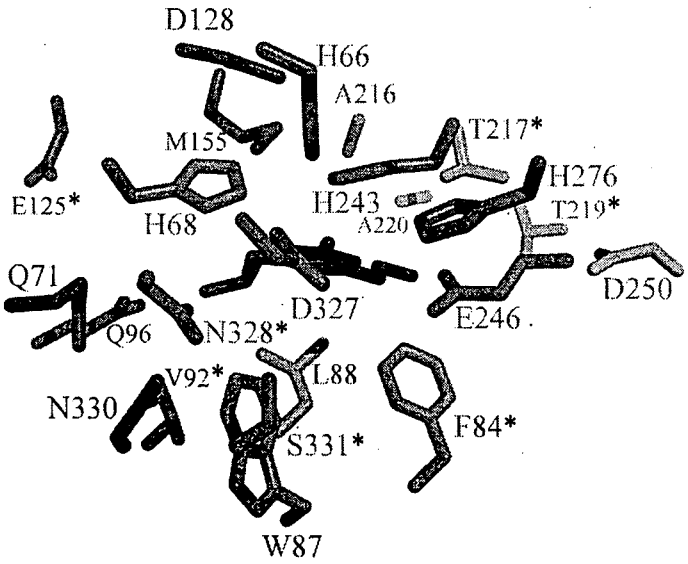


Figure 2b

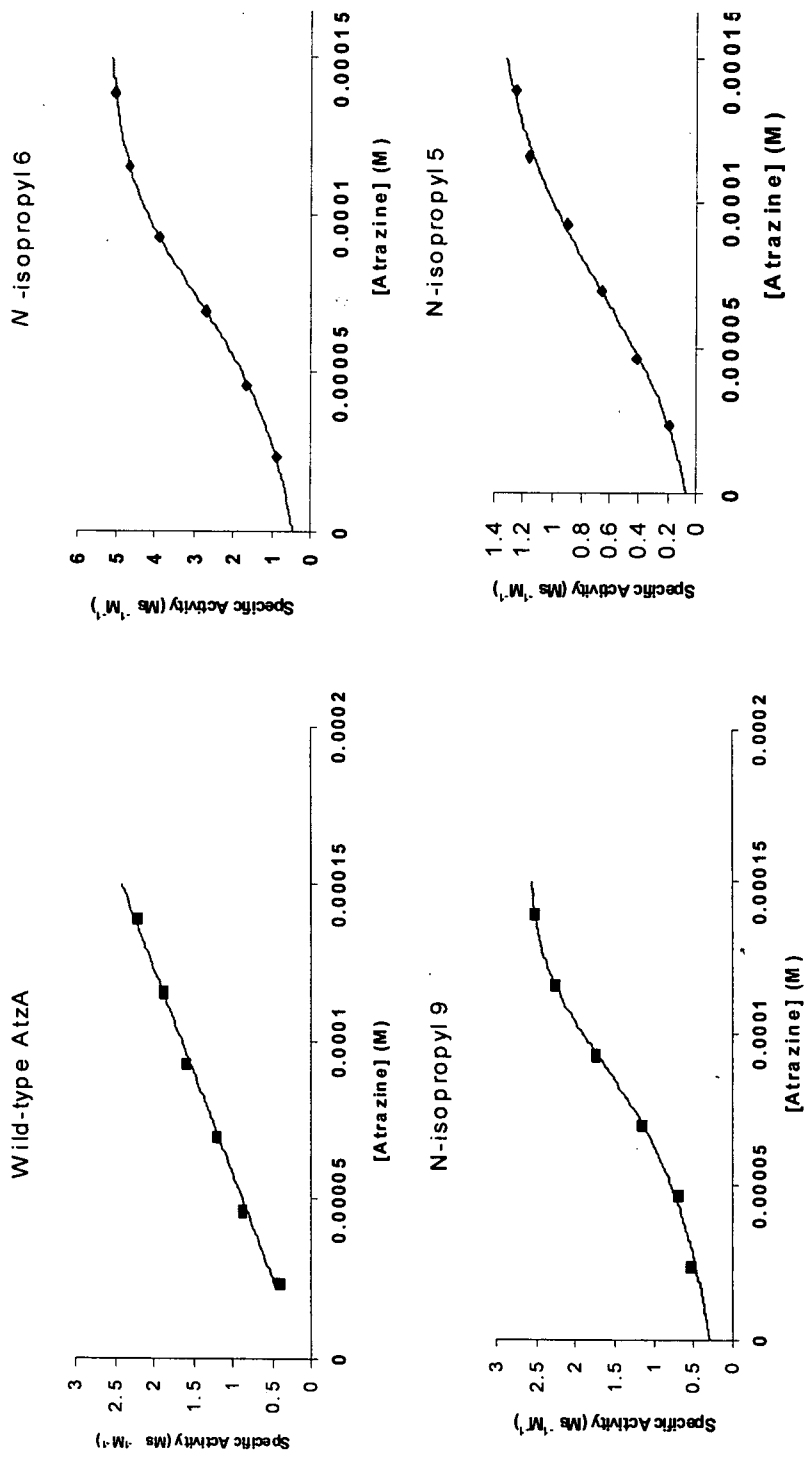

N-isopropyl 6
Specific Activity (Ms -1M-1)
[Atrazine] (M)
N-isopropyl 5
Specific Activity (Ms -1M-1)
[Atrazine] (M)
Wild-type AtzA
Specific Activity (Ms -1M-1)
[Atrazine] (M)
N-isopropyl 9
Specific Activity (Ms -1M-1)
[Atrazine] (M)

Figure 3

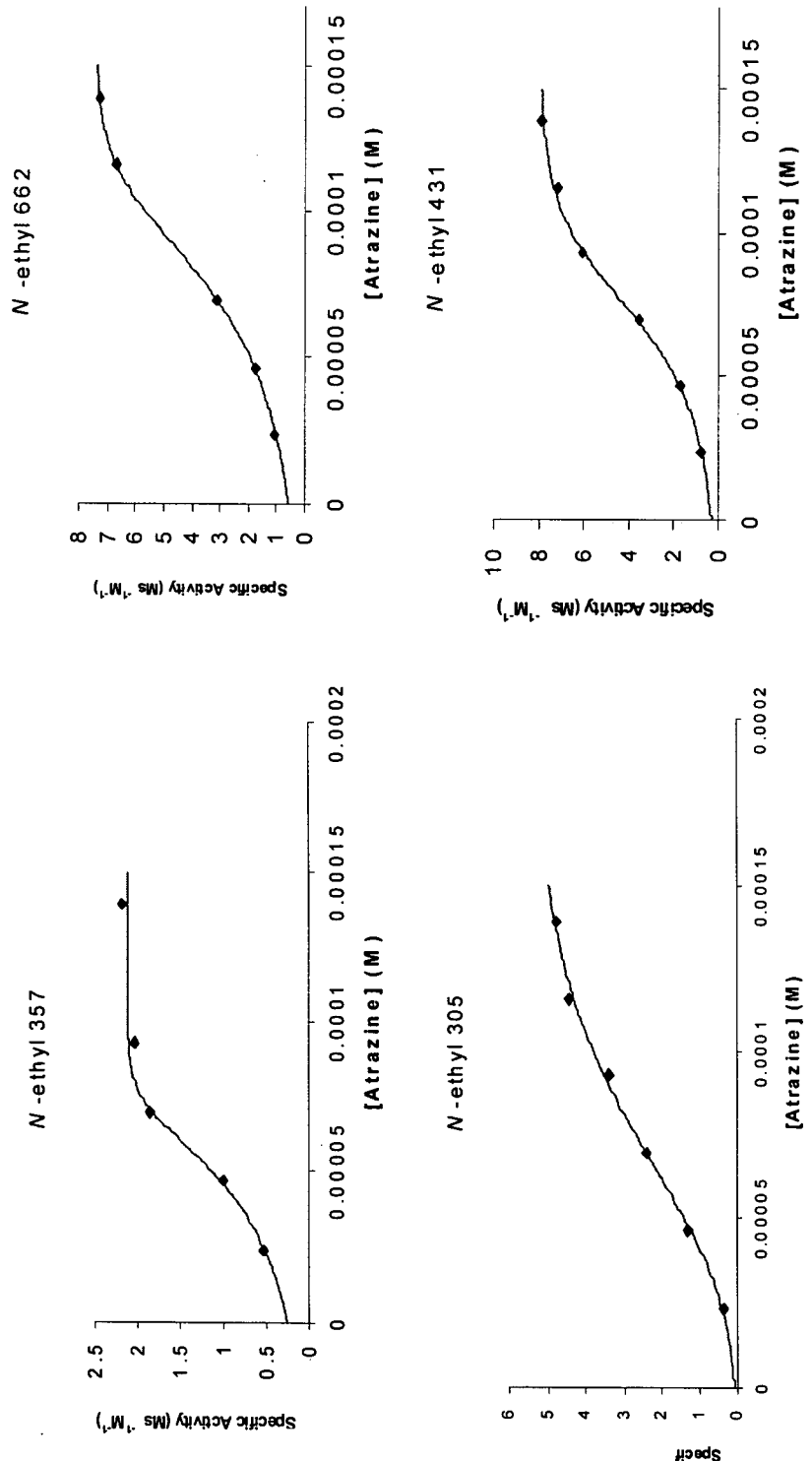


Figure 3 (cont)

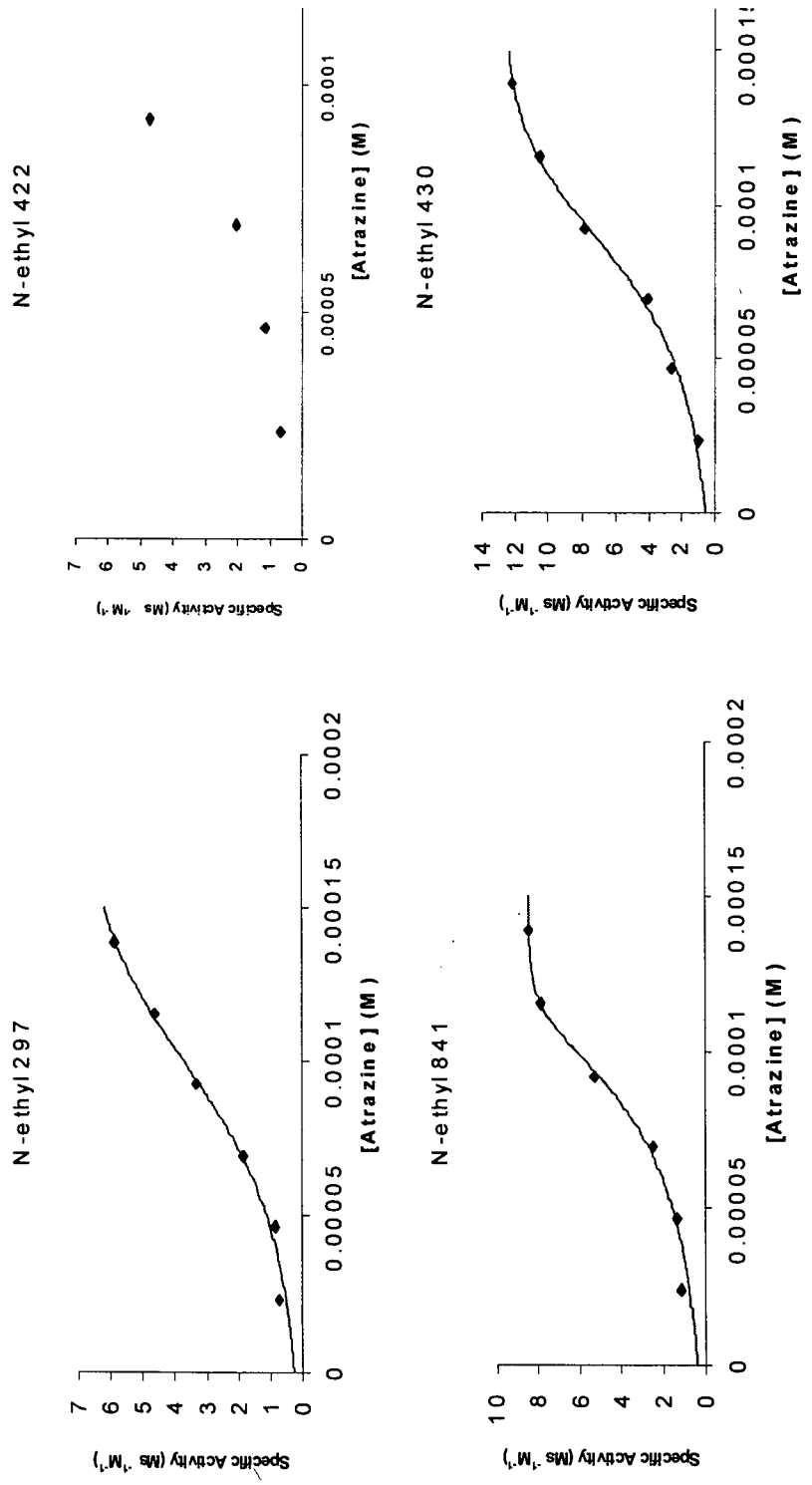
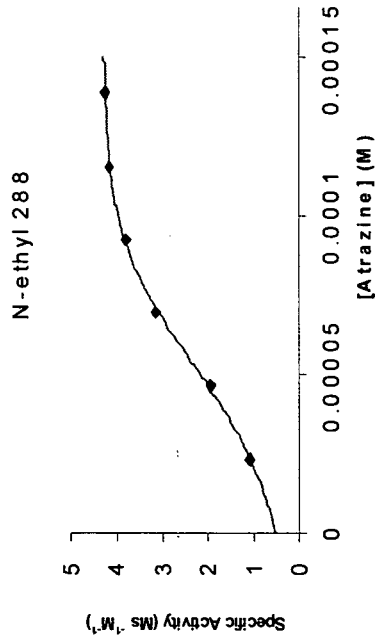
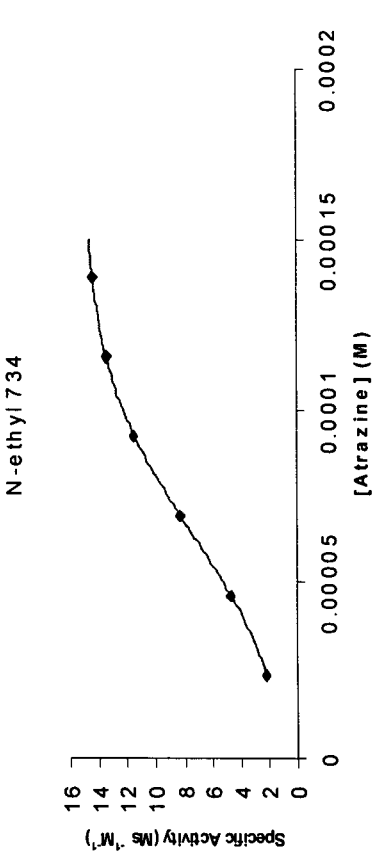


Figure 3 (cont)





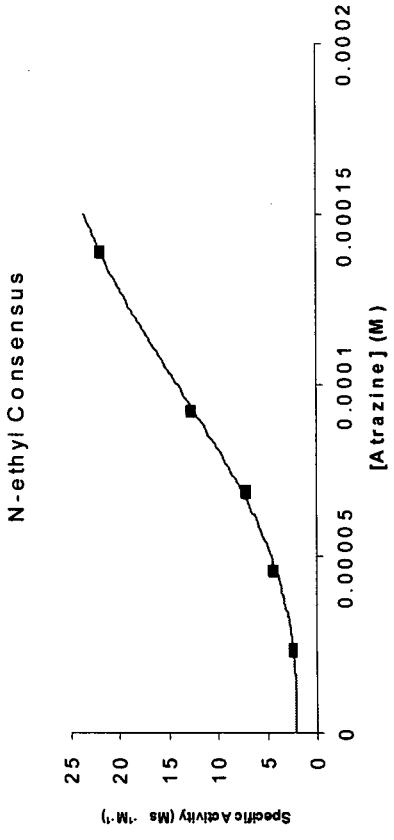


Figure 3 (cont)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001852

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07K 14/195 (2006.01) *B09C 7/70* (2006.01) *C12N 11/08* (2006.01)
A61K 38/46 (2006.01) *C12N 5/10* (2006.01) *C12N 15/55* (2006.01)
A61P 43/00 (2006.01) *C12N 9/14* (2006.01) *C12P 21/00(2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Database: Genomequest; Sequence search based on SEQ ID NO:s 1, 2 and 3

C DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 1997/015675 A1 (REGENTS OF THE UNIVERSITY OF MINNESOTA) 1 May 1997 See SEQ ID NOs: 1 and 2, pages 7, 10, 16-18, 36 and 38 and Figure 8	1, 2, 5, 8, 11, 13-18, 21-24, 30-38
x	US 6,369,299 B1 (SADOWSKY et al.) 9 April 2002 See SEQ ID NOs: 1 and 2, columns 2, 3 and 11	5, 8, 11, 13-19, 21-24, 32, 33
Y	See SEQ ID NOs: 1 and 2, columns 2, 3 and 11	20

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
22 January 2009

Date of mailing of the international search report
20 FEB 2009

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001852

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 1998/03 1816 A 1 (REGENTS OF THE UNIVERSITY OF MINNESOTA) 23 JuIy 1998 See SEQ ID NOs: 17-26, pages 5 and 22-24	5, 8, 11, 13-18, 21-26, 32, 33
X	De Souza, M.L. et al., "Atrazine Chlorohydrolase from *Pseudomonas* sp. Strain ADP: Gene Sequence, Enzyme Purification, and Protein Characterization", *Journal of Bacteriology*, vol. 178, pages 4894-4900 (1996) See Figures 1, 2 and 5, pages 4897 and 4898	1, 5, 8, 11, 13, 14, 22, 23
X	WO 2001/055409 A2 (REGENTS OF THE UNIVERSITY OF MINNESOTA) 2 August 2001 See Figure 8, pages 16, 24 and 31	5, 8, 11, 13-18, 21-29, 32
X	WO 2001/064912 A2 (MAXYGEN, INC.) 7 September 2001 See SEQ ID NOs: 1-96, pages 4, 13-15, 20 and 35	3-5, 7, 8, 10-18, 21-24, 32, 39
Y	Scott, B.B. et al., "Generation of tissue-specific transgenic birds with lentiviral vectors", *PNAS*, vol. 102, pages 16443-16447 (2005) See Methods section	**20**

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2008/001852

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	1997/015675	AU	74669/96	US	6284522	US	2002039778
US	6369299	NONE					
WO	1998/031816	AU	60299/98	US	6265201	US	6825001
		US	2002045236				
WO	2001/055409	AU	31155/01	**us**	6673582	US	6919199
		US	2002098574	**us**	2003170879		
WO	2001/064912	AU	43376/01	EP	1268815	US	2002155571

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX