



(86) Date de dépôt PCT/PCT Filing Date: 1996/04/19
(87) Date publication PCT/PCT Publication Date: 1996/10/24
(45) Date de délivrance/Issue Date: 2012/06/12
(85) Entrée phase nationale/National Entry: 1997/10/17
(86) N° demande PCT/PCT Application No.: US 1996/005782
(87) N° publication PCT/PCT Publication No.: 1996/033270
(30) Priorités/Priorities: 1995/04/20 (US08/426,125);
1995/05/31 (US08/455,355)

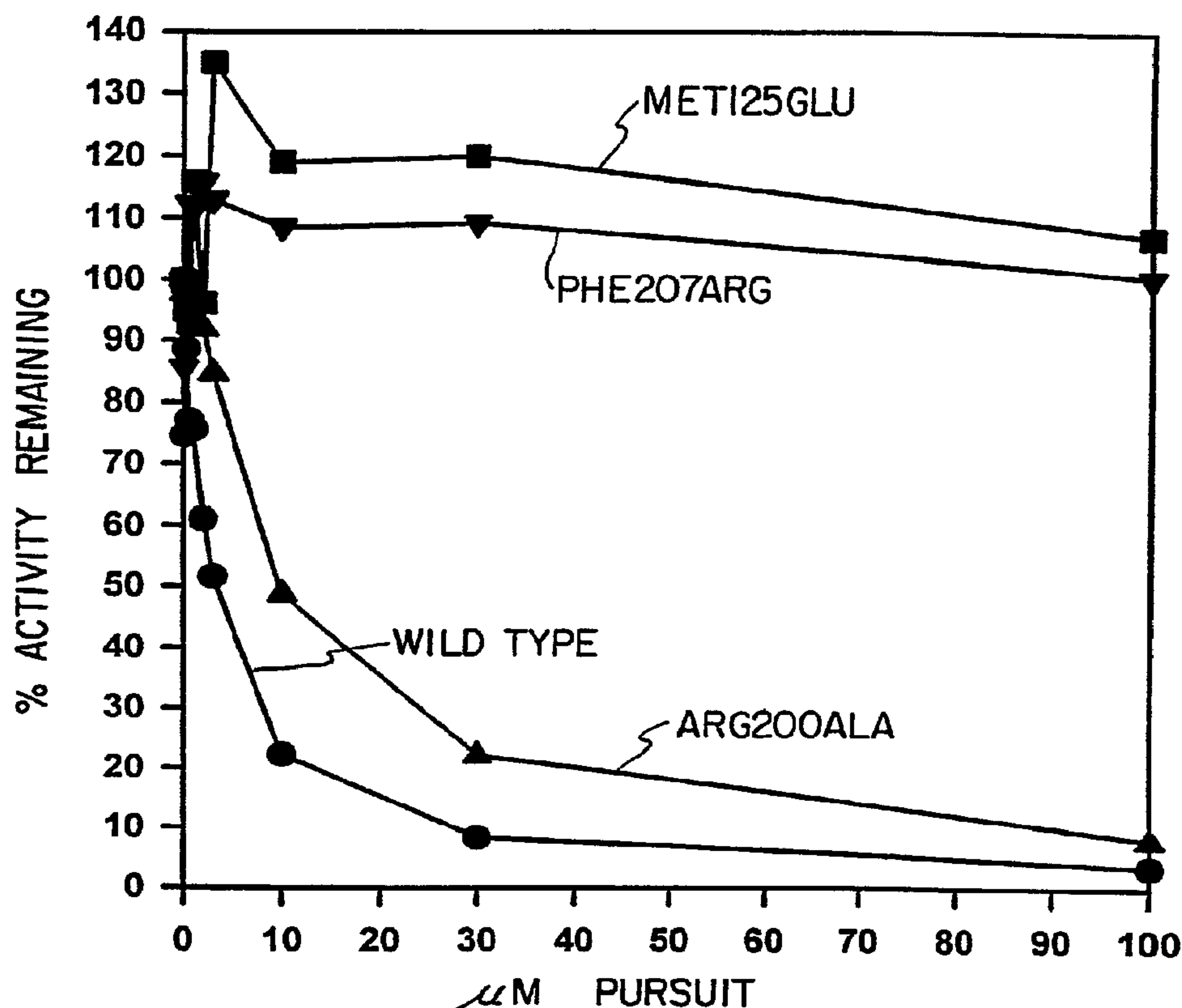
(51) Cl.Int./Int.Cl. *C12N 15/60* (2006.01),
A01H 5/00 (2006.01), *A01H 5/10* (2006.01),
A01N 63/00 (2006.01), *C12N 15/82* (2006.01),
C12N 9/88 (2006.01), *C12Q 1/527* (2006.01)

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(54) Titre : PRODUITS RESISTANT A DES HERBICIDES ELABORES A PARTIR DE STRUCTURES
(54) Title: STRUCTURE-BASED DESIGNED HERBICIDE RESISTANT PRODUCTS



(57) Abrégé/Abstract:

Disclosed herein are structure-based modelling methods for the preparation of acetohydroxy acid synthase (AHAS) variants, including those that exhibit selectively increased resistance to herbicides such as imidazoline herbicides and AHAS inhibiting herbicides. The invention encompasses isolated DNAs encoding such variants, vectors that include the DNAs, and methods for producing the variant polypeptides and herbicide resistant plants containing specific AHAS gene mutations. Methods for weed control in crops are also provided.

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/00, 9/88, A01H 5/00, 5/10		A1	(11) International Publication Number: WO 96/33270
			(43) International Publication Date: 24 October 1996 (24.10.96)
(21) International Application Number: PCT/US96/05782 (22) International Filing Date: 19 April 1996 (19.04.96) (30) Priority Data: 426,125 20 April 1995 (20.04.95) US 455,355 31 May 1995 (31.05.95) US (60) Parent Applications or Grants (63) Related by Continuation US 08/426,125 (CIP) Filed on 20 April 1995 (20.04.95) US 08/455,355 (CIP) Filed on 31 May 1995 (31.05.95) (71) Applicant (for all designated States except US): AMERICAN CYANAMID COMPANY [US/US]; One Cyanamid Plaza, Wayne, NJ 07470-8426 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): KAKEFUDA, Genichi [US/US]; 4 Westover Court, Yardley, PA 19067 (US). OTT, Karl-Heinz [DE/US]; 33 Cypress Court, Lawrenceville, NJ 08648 (US). KWAGH, Jae-Gyu [US/US]; 203 Yorkshire Lane, Fairless Hills, PA 19030 (US). STOCKTON, Gerald,		W. [GB/US]; 391 South Milton Drive, Yardley, PA 19067 (US). (74) Agents: ROBINSON, Joseph, R. et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022 (US). (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(54) Title: STRUCTURE-BASED DESIGNED HERBICIDE RESISTANT PRODUCTS			
(57) Abstract			
Disclosed herein are structure-based modelling methods for the preparation of acetohydroxy acid synthase (AHAS) variants, including those that exhibit selectively increased resistance to herbicides such as imidazoline herbicides and AHAS inhibiting herbicides. The invention encompasses isolated DNAs encoding such variants, vectors that include the DNAs, and methods for producing the variant polypeptides and herbicide resistant plants containing specific AHAS gene mutations. Methods for weed control in crops are also provided.			

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10 **STRUCTURE-BASED DESIGNED HERBICIDE RESISTANT PRODUCTS****Field of the Invention**

15 This invention pertains to structure-based modelling and design of variants of acetohydroxy acid synthase (AHAS) that are resistant to imidazolinones and other herbicides, the AHAS inhibiting herbicides, AHAS variants themselves, DNA encoding these variants, plants expressing these variants, and methods of weed management.

20 **Background of the Invention**

25 Acetohydroxy acid synthase (AHAS) is an enzyme that catalyzes the initial step in the biosynthesis of isoleucine, leucine, and valine in bacteria, yeast, and plants. For example, the mature AHAS from *Zea Mays* is approximately a 599-amino acid protein that is localized in the chloroplast (see Figure 1). The enzyme utilizes thiamine pyrophosphate (TPP) and flavin adenine dinucleotide (FAD) as cofactors and pyruvate as a substrate to form acetolactate. The enzyme also catalyzes the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate. AHAS is also known as acetolactate synthase or acetolactate pyruvate lyase (carboxylating), and is designated EC 4.1.3.18. The active enzyme is probably at least a homodimer. Ibdah et al. (*Protein Science*, 3:479-S, 1994), in an abstract, disclose one model for the active site of AHAS.

35 A variety of herbicides including imidazolinone compounds such as imazethapyr (PURSUIT® - American Cyanamid Company-Wayne, NJ), sulfonylurea-based compounds such as sulfometuron methyl (OUST® - E.I. du Pont de Nemours and

Company-Wilmington, DE), triazolopyrimidine sulfonamides (Broadstrike™ - Dow Elanco; see Gerwick, et al., *Pestic. Sci.* 29:357-364, 1990), sulfamoylureas (Rodaway et al., *Mechanisms of Selectively of Ac 322,140 in Paddy Rice, Wheat and Barley*, Proceedings of the Brighton Crop Protection Conference-Weeds, 1993), pyrimidyl-oxy-benzoic acids (STABLE® - Kumiai Chemical Industry Company, E.I. du Pont de Nemours and Company; see, The Pesticide Manual 10th Ed. pp. 888-889, Clive Tomlin, Ed., British Crop Protection Council, 49 Downing Street, Farmham, Surrey G49 7PH, UNITED KINGDOM), and sulfonylcarboxamides (Alvarado et al., U.S. Patent No. 4,883,914) act by inhibiting AHAS enzymatic activity. (See, Chaleff et al., *Science* 224:1443, 1984; LaRossa et al., *J.Biol.Chem.* 259:8753, 1984; Ray, *Plant Physiol.* 75:827, 11984; Shaner et al., *Plant Physiol.* 76:545, 1984). These herbicides are highly effective and environmentally benign. Their use in agriculture, however, is limited by their lack of selectivity, since crops as well as undesirable weeds are sensitive to the phytotoxic effects of these herbicides.

Bedbrook et al., U.S. Patent Nos. 5,013,659, 5,141,870, and 5,378,824, disclose several sulfonylurea resistant AHAS variants. However, these variants were either obtained by mutagenizing plants, seeds, or cells and selecting for herbicide-resistant mutants, or were derived from such mutants. This approach is unpredictable in that it relies (at least initially) on the random chance introduction of a relevant mutation, rather than a rational design approach based on a structural model of the target protein.

Thus, there is still a need in the art for methods and compositions that provide selective wide spectrum and/or specific herbicide resistance in cultivated crops. The present inventors have discovered that selective herbicide resistant variant forms of AHAS and plants containing the same can be prepared by structure-based modelling of AHAS against pyruvate oxidase (POX), identifying an herbicide binding pocket or pockets on the AHAS model, and designing specific mutations that alter the affinity of the herbicide for the binding pocket. These variants and plants are not inhibited or killed

by one or more classes of herbicides and retain sufficient AHAS enzymatic activity to support crop growth.

Brief Description of the Drawings

5 Figure 1 is an illustration of a 600 amino acid sequence corresponding to the approximately 599 amino acid sequence of acetohydroxy acid synthase (AHAS) from *Zea Mays* which is given as an example of a plant AHAS enzyme. The sequence does not include a transit sequence, and the extra
10 glycine is vestigial from a thrombin cleavage site. Residues Met53, Arg128, and Phe135 are shown in bold.

 Figure 2 is an illustration of the alignment of the sequence of maize AHAS and pyruvate oxidase (POX) from *Lactobacillus planarum*.

15 Figure 3 is a schematic representation of the secondary structure of an AHAS subunit. Regular secondary structure elements, α -helices and β -sheets, are depicted as circles and ellipses, respectively, and are numbered separately for each of the three domains within a subunit. Loops and
20 coiled regions are represented by black lines, with numbers representing the approximate beginnings and ends of the elements. The locations of cofactor binding sites and known mutation sites are indicated by octahedrons and stars, respectively.

25 Figure 4 is an illustration of a computer-generated model of the active site of maize AHAS with imazethapyr (PURSUIT® herbicide) modeled into the binding pocket.

 Figure 5 is an illustration of the homology among AHAS amino acid sequences derived from different plant species.
30 pAC 751 is maize als 2 AHAS isozyme as expressed from the pAC 751 *E. Coli* expression vector as in Figure 1; Maize als 2 is the maize als 2 AHAS isozyme; Maize als 1 is the maize als 1 AHAS isozyme; Tobac 1 is the tobacco AHAS SuRA isozyme; Tobac 2 is the tobacco AHAS SuRB isozyme; Athcsr 12 is the
35 *Arabidopsis thaliana* Csr 1.2 AHAS gene; Bnaal 3 is the *Brassica napus* AHAS III isozyme; and Bnaal 2 is the *Brassica napus* AHAS II isozyme.

pAC 751 and Maize als 2 are identical genes except that Maize als 2 starts at the beginning of the transit sequence and pAC 751 starts at the putative mature N-terminal site with an additional glycine at the N-terminal due to the
5 thrombin recognition sequence in the pGEX-2T expression vector. The N-terminal glycine is not a natural amino acid at that position.

Amino acid sequence alignments of the AHAS proteins were generated by PILEUP (GCG Package - Genetics Computer
10 Group, Inc., - University Research Park - Madison-WI). The consensus sequence was generated by PRETTY GCG Package.

Figure 6 is a photographic illustration of an SDS-polyacrylamide gel stained for protein showing purification of maize AHAS. The lanes contain (from left to right): A,
15 Molecular weight markers; B, Crude *E. coli* cell extract; C, Glutathione-agarose affinity purified preparation; D, Thrombin digest of the affinity purified preparation; E, Second pass through glutathione-agarose column and Sephacryl S-100 gel filtration.

20 Figure 7 is a graphic illustration of the results of *in vitro* assays of the enzymatic activity of wild-type and mutant AHAS proteins in the absence and in the presence of increasing concentrations of imazethapyr (PURSUIT® herbicide). The Y axis represents the % of activity of the mutant enzyme,
25 wherein the 100% value is measured in the absence of inhibitor.

Figure 8 is a graphic illustration of the results of *in vitro* assays of the enzymatic activity of wild-type and mutant AHAS proteins in the absence and presence of increasing concentrations of sulfometuron methyl (OUST® herbicide). The
30 Y axis represents the % of activity of the mutant enzyme, wherein the 100% value is measured in the absence of inhibitor.

Figure 9 is a graphic illustration of *in vitro* assays of the enzymatic activity of wild-type *Arabidopsis* AHAS protein and the Met124Ile mutant *Arabidopsis* AHAS protein in the
35 absence and presence of increasing concentrations of imazethapyr (PURSUIT® herbicide) and sulfometuron methyl (OUST® herbicide). The Y axis represents the % activity of the mutant

*Trade-mark

enzyme, wherein the 100% value is measured in the absence of inhibitor.

Figure 10 is a graphic illustration of *in vitro* assays of the enzymatic activity of wild-type *Arabidopsis* AHAS protein and the Met124His mutant *Arabidopsis* AHAS protein in the absence and presence of increasing concentrations of imazethapyr (PURSUIT® herbicide) and sulfometuron methyl (OUST® herbicide). The Y axis represents the % activity of the mutant enzyme, wherein the 100% value is measured in the absence of inhibitor.

Figure 11 is a graphic illustration of *in vitro* assays of the enzymatic activity of wild-type *Arabidopsis* AHAS protein and Arg199Glu mutant *Arabidopsis* AHAS protein in the absence and presence of increasing concentrations of imazethapyr (PURSUIT® herbicide) and sulfometuron methyl (OUST® herbicide). The Y axis represents the % activity of the mutant enzyme, wherein the 100% value is measured in the absence of inhibitor.

Figure 12 is a schematic illustration of a DNA vector used for plant transformation, which contains the nptII gene (encoding kanamycin resistance) under the control of the 35S promoter and an AHAS gene (wild type or variant) under the control of the *Arabidopsis* AHAS promoter.

Figure 13 is a photograph showing the root development of tobacco plants transformed with the *Arabidopsis* AHAS gene containing either the Met124Ile or Arg199Glu mutation and a non-transformed control. Plants were grown for 18 days after transfer into medium containing 0.25 μ M imazethapyr.

Figure 14 is a photograph showing tobacco plants transformed with the *Arabidopsis* AHAS gene containing either the Met124Ile, Met 124His, or Arg199Glu mutation and a non-transformed control, which had been sprayed with twice the field rate (100 g/ha) of imazethapyr.

Figure 15 is a photograph showing the results of germination tests performed in the presence of the herbicide CL 299,263 (imazamox), which were performed on seeds harvested from primary tobacco plant transformants that had been

transformed with the *Arabidopsis* AHAS gene containing either the Met124Ile, Met 124His, or Arg199Glu mutation.

Summary of the Invention

5 The present invention provides a structure-based modelling method for the production of herbicide resistant AHAS variant protein. The method includes:

10 (a) aligning a target AHAS protein on pyruvate oxidase template or an AHAS modelling equivalent thereof to derive the three-dimensional structure of the target AHAS protein;

 (b) modelling one or more herbicides into the three-dimensional structure to localize an herbicide binding pocket in the target AHAS protein;

15 (c) selecting as a target for a mutation, at least one amino acid position in the target AHAS protein, wherein the mutation alters the affinity of at least one herbicide for the binding pocket;

20 (d) mutating DNA encoding the target AHAS protein to produce a mutated DNA encoding a variant AHAS containing the mutation, such as, for example, at least one different amino acid, at the position; and

25 (e) expressing the mutated DNA in a first cell, under conditions in which the variant AHAS containing the mutation, such as, for example, the different amino acid(s), at the position is produced.

 The method further may include:

 (f) expressing DNA encoding wild-type AHAS protein parallel in a second cell;

30 (g) purifying the wild-type and the variant AHAS proteins from the cells;

 (h) assaying the wild-type and the variant AHAS proteins for catalytic activity in conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetoxybutyrate, in the absence and
35 in the presence of the herbicide; and

 (i) repeating steps (c)-(h), wherein the DNA encoding the AHAS variant of step (e) is used as the AHAS-

encoding DNA in step (c) until a first herbicide resistant AHAS variant protein is identified having:

(i) in the absence of the at least one herbicide,

5 (a) catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(b) catalytic activity in combination with any herbicide resistant AHAS variant protein also
10 expressed in the cell, which may be the same as or different than the first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein the cell requires AHAS activity for viability; and

15 (ii) catalytic activity that is more resistant to the at least one herbicide than is wild-type AHAS.

An alternate structure-based modelling method for the production of herbicide resistant AHAS variant protein is also
20 provided. This method includes:

(a) aligning a target AHAS protein on a first AHAS template derived from a polypeptide having the sequence of Figure 1 or a functional equivalent thereof to derive the three-dimensional structure of the target AHAS protein;

25 (b) modelling one or more herbicides into the three-dimensional structure to localize an herbicide binding pocket in the target AHAS protein;

(c) selecting as a target for a mutation, at least one amino acid position in the target AHAS protein,
30 wherein the mutation alters the affinity of at least one herbicide for the binding pocket;

(d) mutating DNA encoding the target AHAS protein to produce a mutated DNA encoding a variant AHAS containing the mutation at the position; and

35 (e) expressing the mutated DNA in a first cell, under conditions in which the variant AHAS containing the mutation at the position is produced.

This method can further include:

- (f) expressing DNA encoding wild-type AHAS protein in parallel in a second cell;
- (g) purifying the wild-type and the variant AHAS protein from the cells;
- 5 (h) assaying the wild-type and the variant AHAS protein for catalytic activity in conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of the herbicide; and
- 10 (i) repeating steps (c)-(h), wherein the DNA encoding the AHAS variant of step (e) is used as the AHAS-encoding DNA in step (c) until a first herbicide resistant AHAS variant protein is identified having:
- (i) in the absence of the at least one
- 15 herbicide,
- (a) catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or
- (b) catalytic activity in combination
- 20 with any herbicide resistant AHAS variant protein also expressed in the cell, which may be the same as or different than the first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;
- wherein the cell requires AHAS
- 25 activity for viability; and
- (ii) catalytic activity that is more resistant to the at least one herbicide than is wild-type AHAS.

In another alternate embodiment, the method includes:

- 30 (a) aligning a target AHAS protein on a first AHAS template having an identified herbicide binding pocket and having the sequence of Figure 1 or a functional equivalent thereof to derive the three-dimensional structure of the target AHAS protein;
- 35 (b) selecting as a target for a mutation, at least one amino acid position in the target AHAS protein, wherein the mutation alters the affinity of at least one herbicide for the binding pocket;

(c) mutating DNA encoding the target AHAS protein to produce a mutated DNA encoding a variant AHAS containing the mutation at the position; and

(d) expressing the mutated DNA in a first cell,
5 under conditions in which the variant AHAS containing the mutation at the position is produced.

This method can further include:

(e) expressing DNA encoding wild-type target AHAS protein in parallel in a second cell;

10 (f) purifying the wild-type and the variant AHAS protein from the cells;

(g) assaying the wild-type and the variant AHAS protein for catalytic activity in conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-
15 ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of the herbicide; and

(h) repeating steps (b)-(g), wherein the DNA encoding the AHAS variant of step (d) is used as the AHAS-
-- encoding DNA in step (b) until a first herbicide resistant AHAS
20 variant protein is identified having:

(i) in the absence of the at least one herbicide,

(a) catalytic activity alone sufficient to maintain the viability of a cell in which it is
25 expressed; or

(b) catalytic activity in combination with any herbicide resistant AHAS variant protein also expressed in the cell, which may be the same as or different than the first AHAS variant protein, sufficient to maintain the
30 viability of a cell in which it is expressed;

wherein the cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to the at least one herbicide than is wild-type AHAS.

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In preferred embodiments of the above methods, the catalytic activity in the absence of the herbicide is at least about 5% and most preferably is more than about 20% of the

catalytic activity of the wild-type AHAS. Where the herbicide is an imidazolinone herbicide, the herbicide resistant AHAS variant protein preferably has:

(i) catalytic activity in the absence of the herbicide of more than about 20% of the catalytic activity of the wild-type AHAS;

(ii) catalytic activity that is relatively more resistant to the presence of imidazolinone herbicides compared to wild-type AHAS; and

(iii) catalytic activity that is relatively more sensitive to the presence of sulfonylurea herbicides compared to imidazolinone herbicides.

The present invention further provides isolated DNA encoding acetohydroxy acid synthase (AHAS) variant proteins, the variant proteins comprising an AHAS protein modified by:

(i) substitution of at least one different amino acid residue at an amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(ii) deletion of up to 5 amino acid residues preceding, or up to 5 amino acid residues following at least one amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261,

M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(iii) deletion of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(iv) addition of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(v) deletion of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1;

(vi) addition of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1; or

(vii) any combination of any of the foregoing.

In this numbering system, residue #2 corresponds to the putative amino terminus of the mature protein, i.e., after removal of a chloroplast targeting peptide.

The above modifications are directed to altering the ability of an herbicide, and preferably an imidazolinone-based herbicide, to inhibit the enzymatic activity of the protein. In a preferred embodiment, the isolated DNA encodes an herbicide-resistant variant of AHAS. Also provided are DNA vectors comprising DNA encoding these AHAS variants, variant AHAS proteins themselves, and cells, grown either *in vivo* or in cell culture, that express the AHAS variants or comprise these vectors.

In another aspect, the present invention provides a method for conferring herbicide resistance on a cell or cells and particularly a plant cell or cells such as, for example, a seed. An AHAS gene, preferably the *Arabidopsis thaliana* AHAS gene, is mutated to alter the ability of an herbicide to inhibit the enzymatic activity of the AHAS. The mutant gene is cloned into a compatible expression vector, and the gene is transformed into an herbicide-sensitive cell under conditions in which it is expressed at sufficient levels to confer herbicide resistance on the cell.

Also contemplated are methods for weed control, wherein a crop containing an herbicide resistant AHAS gene according to the present invention is cultivated and treated with a weed-controlling effective amount of the herbicide.

Also disclosed is a structure-based modelling method for the preparation of a first herbicide which inhibits AHAS activity. The method comprises:

(a) aligning a target AHAS protein on pyruvate oxidase template or an AHAS modelling functional equivalent thereof to derive the three-dimensional structure of the target AHAS protein;

(b) modelling a second herbicide having AHAS inhibiting activity into the three-dimensional structure to derive the location, structure, or a combination thereof of an herbicide binding pocket in the target AHAS protein; and

(c) designing a non-peptidic first herbicide which will interact with, and preferably will bind to, an AHAS activity inhibiting effective portion of the binding pocket, wherein the first herbicide inhibits the AHAS activity sufficiently to destroy the viability of a cell which requires AHAS activity for viability.

An alternative structure-based modelling method for the production of a first herbicide which inhibits AHAS activity, is also enclosed. The method comprises:

(a) aligning a target AHAS protein on a first AHAS template derived from a polypeptide having the sequence of Figure 1 or a functional equivalent thereof, to derive the three-dimensional structure of the target AHAS protein;

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(b) modelling a second herbicide having AHAS inhibiting activity into the three-dimensional structure to derive the location, structure, or a combination thereof of a herbicide binding pocket in the target AHAS protein; and

5 (c) designing a non-peptidic first herbicide which will interact with, and preferably will bind to, an AHAS activity inhibiting effective portion of the binding pocket, wherein the first herbicide inhibits the AHAS activity sufficiently to destroy the viability of a cell which
10 requires AHAS activity for viability.

Preferably in each method, the first herbicide contains at least one functional group that interacts with a functional group of the binding pocket.

Thus, in one aspect, the invention relates to a
15 method for the production of an imidazolinone and/or sulfonylurea based resistant acetohydroxy acid synthase (AHAS) variant protein using structure-based information, said method comprising: (a) aligning a target AHAS protein on pyruvate oxidase template to derive the three-dimensional
20 structure of said target AHAS protein; (b) modelling one or more imidazolinone and/or sulfonylurea based herbicides into said three-dimensional structure to localize an imidazolinone and/or sulfonylurea based herbicide binding pocket in said target AHAS protein; (c) selecting as a target for a mutation
25 an amino acid position in said target AHAS protein, wherein said mutation alters the affinity of at least one imidazolinone and/or sulfonylurea based herbicide for said binding pocket; (d) mutating DNA encoding said target AHAS protein to produce a mutated DNA encoding a variant AHAS

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containing said mutation at said position; and (e) expressing said mutated DNA in a first cell, under conditions in which said variant AHAS containing said mutation at said position is produced.

5 In another aspect, the invention relates to a method for the production of an imidazolinone and/or sulfonylurea based resistant acetohydroxy acid synthase (AHAS) variant protein using structure-based information, said method comprising: (a) aligning a target AHAS protein
10 on a first AHAS template derived from a polypeptide having the sequence of SEQ ID NO: 1 or the amino acid sequence of another plant AHAS protein, to derive the three-dimensional structure of said target AHAS protein; (b) modelling one or more imidazolinone or sulfonylurea based herbicides into
15 said three-dimensional structure to localize an imidazolinone or sulfonylurea based herbicide binding pocket in said target AHAS protein; (c) selecting as a target for a mutation, at least one amino acid position in said target AHAS protein, wherein said mutation alters the affinity of
20 at least one imidazolinone or sulfonylurea based herbicide for said binding pocket; (d) mutating DNA encoding said target AHAS protein to produce a mutated DNA encoding a variant AHAS containing the mutation at said position; and
25 (e) expressing said mutated DNA in a first cell, under conditions in which said variant AHAS containing said mutation at said position is produced.

 In another aspect, the invention relates to a method for the production of imidazolinone and/or sulfonylurea based herbicide resistant acetohydroxy acid
30 synthase (AHAS) variant protein using structure-based

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information, said method comprising: (a) aligning a target AHAS protein on a first AHAS template having an identified imidazolinone and/or sulfonylurea based herbicide binding pocket and having the sequence of SEQ ID NO: 1 to derive the
5 three-dimensional structure of said target AHAS protein;
(b) selecting as a target for a mutation, at least one amino acid position in said target AHAS protein, wherein said mutation alters the affinity of an imidazolinone or sulfonylurea based herbicide for said binding pocket;
10 (c) mutating DNA encoding said target AHAS protein to produce a mutated DNA encoding a variant AHAS containing said mutation at said position; and (d) expressing said mutated DNA in a first cell, under conditions in which said variant AHAS containing said mutation at said position is
15 produced.

In another aspect, the invention relates to an isolated DNA encoding an acetohydroxy acid synthase (AHAS) variant protein, said variant protein comprising an AHAS protein modified by substitution of at least one different
20 amino acid residue at an amino acid residue of the sequence of SEQ ID NO: 1 selected from the group consisting of M53, R128, F135, and any combination of any of the foregoing.

In another aspect, the invention relates to a DNA vector comprising the DNA sequence as described above
25 operably linked to a transcription regulatory element.

In another aspect, the invention relates to a cell comprising an acetohydroxy acid synthase (AHAS) encoding DNA sequence derived from the DNA vector as described above, wherein said cell is selected from the group consisting of
30 bacterial, fungal, plant, insect, and mammalian cells.

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In another aspect, the invention relates to use of the cell as described above for the production of a seed.

In another aspect, the invention relates to a variant acetohydroxy acid synthase (AHAS) protein comprising
5 a protein encoded by the isolated DNA as described above.

In another aspect, the invention relates to a variant acetohydroxy acid synthase (AHAS) protein comprising an AHAS protein modified by substitution of at least one different amino acid residue at an amino acid residue of the
10 sequence of SEQ ID NO: 1 selected from the group consisting of M53, R128, F135, an amino acid residue of another plant AHAS protein at an amino acid aligned with any of the foregoing, and any combination of any of the foregoing.

In another aspect, the invention relates to a
15 method for conferring imidazolinone and/or sulfonylurea based herbicide resistance on a cell, said method comprising: (a) cloning the isolated DNA as described above into a compatible expression vector; and (b) transforming said DNA into said cell, under conditions wherein said gene
20 is expressed at sufficient levels to confer imidazolinone or sulfonylurea based herbicide resistance on said cell.

In another aspect, the invention relates to a cell prepared according to the method as described above.

In another aspect, the invention relates to use of
25 a cell as described above for the production of a plant.

In another aspect, the invention relates to a method for controlling weeds in a crop, said method comprising cultivating a crop comprising an imidazolinone

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13d

and/or sulfonylurea based herbicide resistant plant comprising the cell as described above, and treating said crop with said imidazolinone and/or sulfonylurea based herbicide.

5 In another aspect, the invention relates to a method for controlling weeds in a crop, said method comprising cultivating a crop comprising an imidazolinone and/or sulfonylurea based herbicide resistant plant comprising the cell as described above, and treating said
10 crop with said imidazolinone and/or sulfonylurea based herbicidal composition including said herbicide.

 In another aspect, the invention relates a cell transformed with the DNA as described above, wherein said DNA is expressed in said cell such that it confers
15 imidazolinone and/or sulfonylurea based herbicide resistance on said cell.

 In another aspect, the invention relates to use of a cell comprising the DNA as described above for the production of an imidazolinone and/or sulfonylurea based
20 herbicide resistant plant.

 In another aspect, the invention relates to use of a plant comprising a cell transformed with the DNA as described above for the production of an imidazolinone and/or sulfonylurea based herbicide resistant plant.

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Detailed Description of the Invention

The present invention encompasses the rational design or structure-based molecular modelling of modified versions of the enzyme AHAS and AHAS inhibiting herbicides. These modified enzymes (AHAS variant proteins) are resistant to the action of herbicides. The present invention also encompasses DNAs that encode these variants, vectors that include these DNAs, the AHAS variant proteins, and cells that express these variants. Additionally provided are methods for producing herbicide resistance in plants by expressing these variants and methods of weed control. The DNA and the AHAS variants of the present invention were discovered in studies that were based on molecular modelling of the structure of AHAS.

15 Rational Structure-Based Design of AHAS Variants and AHAS Inhibiting Herbicides

Herbicide-resistant variants of AHAS according to the present invention are useful in conferring herbicide resistance in plants and can be designed with the POX model, AHAS model, or functional equivalents thereof, such as, for example, transketolases, carboligases, pyruvate decarboxylase, proteins that bind FAD and/or TPP as a cofactor, or any proteins which have structural features similar to POX and/or AHAS; with an AHAS model such as a model having the sequence of Figure 1; or

with a functional equivalent of the sequence of Figure 1 including a variant modeled from a previous model. Proteins that can be used include any proteins having less than a root mean square deviation of less than 3.5 angstroms in their C α carbons relative to any of the above-listed molecules. AHAS directed herbicides can be similarly modelled from these templates. A functional equivalent of an AHAS amino acid sequence is a sequence having substantial, i.e., 60-70%, homology, particularly in conserved regions such as, for example, a putative binding pocket. The degree of homology can be determined by simple alignment based on programs known in the art, such as, for example, GAP and PILEUP by GCG. Homology means identical amino acids or conservative substitutions. A functional equivalent of a particular amino acid residue in the AHAS protein of Figure 1 is an amino acid residue of another AHAS protein which when aligned with the sequence of Figure 1 by programs known in the art, such as, for example, GAP and PILEUP by GCG, is in the same position as the amino acid residue of Figure 1.

Rational design steps typically include: (1) alignment of a target AHAS protein with a POX backbone or structure or an AHAS backbone or structure; (2) optionally, and if the AHAS backbone has an identified herbicide binding pocket, modelling one or more herbicides into the three-dimensional structure to localize an herbicide binding pocket in the target protein; (3) selection of a mutation based upon the model; (4) site-directed mutagenesis; and (5) expression and purification of the variants. Additional steps can include (6) assaying of enzymatic properties and (7) evaluation of suitable variants by comparison to the properties of the wild-type AHAS. Each step is discussed separately below.

1. Molecular Modelling

Molecular modelling (and particularly protein homology modelling) techniques can provide an understanding of the structure and activity of a given protein. The structural model of a protein can be determined directly from experimental data such as x-ray crystallography, indirectly by homology

modelling or the like, or combinations thereof (See White, et al., *Annu. Rev. Biophys. Biomol. Struct.*, 23:349, 1994). Elucidation of the three-dimensional structure of AHAS provides a basis for the development of a rational scheme for mutation of particular amino acid residues within AHAS that confer herbicide resistance on the polypeptide.

Molecular modelling of the structure of *Zea mays* AHAS, using as a template the known X-ray crystal structure of related pyruvate oxidase (POX) from *Lactobacillus plantarum*, provides a three-dimensional model of AHAS structure that is useful for the design of herbicide-resistant AHAS variants or AHAS inhibiting herbicides. This modelling procedure takes advantage of the fact that AHAS and POX share a number of biochemical characteristics and may be derived from a common ancestral gene (Chang et al., *J. Bacteriol.* 170:3937, 1988).

Because of the high degree of cross-species homology in AHAS the modelled AHAS described herein or functional equivalents thereof can also be used as templates for AHAS variant protein design.

Derivation of one model using interactive molecular graphics and alignments is described in detail below. The three-dimensional AHAS structure that results from this procedure predicts the approximate spatial organization of the active site of the enzyme and of the binding site or pocket of inhibitors such as herbicides including, but not limited to, imidazolinone herbicides. The model is then refined and re-interpreted based on biochemical studies which are also described below.

Protein homology modelling requires the alignment of the primary sequence of the protein under study with a second protein whose crystal structure is known. Pyruvate oxidase (POX) was chosen for AHAS homology modelling because POX and AHAS share a number of biochemical characteristics. For example, both AHAS and POX share aspects of enzymatic reaction mechanisms, as well as cofactor and metal requirements. In both enzymes thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), and a divalent cation are required for enzymatic activity. FAD mediates a redox reaction during

catalysis in POX but presumably has only a structural function in AHAS, which is possibly a vestigial remnant from the evolution of AHAS from POX. Both enzymes utilize pyruvate as a substrate and form hydroxyethyl thiamine pyrophosphate as a stable reaction intermediate (Schloss, J.V. et al. In *Biosynthesis of branched chain amino acids*, Barak, Z.J.M., Chipman, D.M., Schloss, J.V. (eds) VCH Publishers, Weinheim, Germany, 1990).

Additionally, AHAS activity is present in chimeric POX-AHAS proteins consisting of the N-terminal half of POX and the C-terminal half of AHAS, and there is a small degree of AHAS activity exhibited by POX itself. AHAS and POX also exhibit similar properties in solution (Risse, B. et al, *Protein Sci.* 1: 1699 and 1710, 1992; Singh, B.K., & Schmitt, G.K. (1989), *FEBS Letters*, 258: 113; Singh, B.K. et al. (1989) In: *Prospects for Amino Acid Biosynthesis Inhibitors in Crop Protection and Pharmaceutical Chemistry*, (Lopping, L.G., et al., eds., BCPC Monograph p. 87). With increasing protein concentration, both POX and AHAS undergo stepwise transitions from monomers to dimers and tetramers. Increases in FAD concentration also induce higher orders of subunit assembly. The tetrameric form of both proteins is most stable to heat and chemical denaturation.

Furthermore, the crystal structure of POX from *Lactobacillus planarum* had been solved by Muller et al., *Science* 259:965, 1993. The present inventors found that based in part upon the degree of physical, biochemical, and genetic homology between AHAS and POX, the X-ray crystal structure of POX could be used as a structural starting point for homology modelling of the AHAS structure.

AHAS and *L. plantarum* POX sequences were not similar enough for a completely computerized alignment, however. Overall, only about 20% of the amino acids are identical, while about 50% of the residues are of similar class (i.e. acidic, basic, aromatic, and the like). However, if the sequences are compared with respect to hydrophilic and hydrophobic residue classifications, over 500 of the 600 amino acids match. Secondary structure predictions for AHAS (Holley et al.,

Proc.Natl.Acad.Sci.USA 86:152, 1989) revealed a strong similarity to the actual secondary structure of POX. For nearly 70% of the residues, the predicted AHAS secondary structure matches that of POX.

5 POX monomers consist of three domains, all having a central, parallel β -sheet with crossovers consisting of α -helices and long loops. (Muller et al., *Science* 259:965, 1993). The topology of the sheets differs between the domains, i.e. in the first and third domains, the strands are assembled
10 to the β -sheet in the sequence 2-1-3-4-6-5, while in the β -sheet of the second domain, the sequence reads 3-2-1-4-5-6.

Computer generated alignments were based on secondary structure prediction and sequence homology. The conventional pair-wise sequence alignment method described by Needleman and
15 Wunch, *J. Mol. Biol*, 48: 443, 1970, was used. Two sequences were aligned to maximize the alignment score. The alignment score (homology score) is the sum of the scores for all pairs of aligned residues, plus an optional penalty for the introduction of gaps into the alignment. The score for the
20 alignment of a pair of residues is a tabulated integer value. The homology scoring system is based on observing the frequency of divergence between a given pair of residues. (MO Dayhoff, RM Schwartz & BC Orcutt "Atlas of Protein Sequence and Structure" vol. 5 suppl. 3 pp. 345-362, 1978).

25 The alignments were further refined by repositioning gaps so as to conserve continuous regular secondary structures. Amino acid substitutions generated by evaluation of likely alignment schemes were compared by means of interactive molecular graphics. Alignments with the most conservative
30 substitutions with respect to the particular functionality of the amino acids within a given site were chosen. The final alignment of both POX and AHAS is displayed in Figure 2. Conserved clusters of residues were identified, in particular for the TPP binding site and for parts of the FAD binding site.
35 The alignment revealed a high similarity between AHAS and POX for the first domain, for most parts of the second domain, and for about half of the third domain. Most of the regions that aligned poorly and may fold differently in POX and in AHAS were

expected to be at the surface of the protein and were not involved in cofactor or inhibitor binding. The prediction of mutation sites is not substantially affected by small shifts in the alignment.

5 Most TPP binding residues are highly conserved between POX and AHAS (e.g. P48-G49-G50). In some cases, residues that were close to TPP differ between POX and AHAS but remain within a region that is highly conserved (for example, residues 90-110). On the other hand, the FAD binding site
10 appeared to be less conserved. Although some FAD binding residues were strongly conserved (for example, D325-I326-D327-P328), others clearly differed between AHAS and POX (for example, residues in the loop from positions 278 to 285 are not homologous. A detailed analysis revealed that, at least for
15 some of the less-conserved contact sites, the interactions were mediated by the polypeptide backbone rather than by the side chains. Hence, conservation was only required for the polypeptide fold and was not required for the amino acid sequence (for example, the backbone of residues 258-263 binds
20 the ribitol chain of FAD). One half of the adenine and the isoalloxazine binding sites clearly differ.

After aligning the primary structure, a homology model was built by transposition of AHAS amino acid sequences to the POX template structure. Missing coordinates were built
25 stepwise using templates of amino acid residues to complete undefined side chains. Data bank searches and energy minimization of small parts of the molecule were used to complete the conformations of undefined loop regions. The cofactors TPP and FAD were modeled into their binding pockets.
30 This model was then subjected to a complete, 5000 cycle energy minimization. All computer modelling was performed in an IRIS Indigo Elan^{*} R4000 Workstation from Silicon Graphics^{*} Co. Interactive molecular modelling and energy-minimization were performed using Quanta/CHARMM^{*} 4.0 from Molecular Simulations
35 Inc. During this step, the conformation was stable, indicating that no strongly disfavored interactions, such as, for example, close van der Waals contacts, had occurred. The results are shown schematically in Figure 3.

^{*}Trade-mark

Characteristics of Predicted AHAS Structure

Inspection of the modelled AHAS structure described above revealed that most of the protein folds with a backbone that is energetically reasonable, with most hydrophilic side chains accessible to the solvent. The surface of the β -sheets are smooth and accommodate the cross-over regions that are attached to them.

A model for dimeric AHAS was generated by duplicating the coordinates of the energy minimized monomeric AHAS and superimposing the two copies on two POX subunits using pairs of C α coordinates as defined in the alignment scheme. The polypeptide chain of AHAS folds into three similarly folded domains composed of a six-stranded parallel β -sheet core surrounded by long "loops" and α -helices. Two subunits are assembled such that the first domain of one subunit is in close proximity to the cofactor-binding domains 2 and 3 of the other subunit. A solvent-filled space remains between the subunits at this site. This pocket, which is defined by the confluence of the three domains, is the proposed entry site for the substrate. It is also proposed to be the binding site for herbicides.

The inner surface of the binding pocket is outlined by the cofactors. The thiazol of TPP is positioned at the bottom of the pocket. Domain 3 contributes to the inner surface of the pocket with a short α -helix that points its axis towards the pyrophosphate of TPP, compensating the phosphate charges with its dipolar moment. This critical helix, which starts with G498, a "turn" residue in close contact with TPP, and which ends at F507, contains three known mutation sites for sulfonylurea resistance: V500, W503, and F507 (See, U.S. Patent Nos. 5,013,659; 5,141,870; and 5,378,824). In domain 1, the loop defined as P48-S52 (between β -strand 2 and α -helix 2) faces W503, a mutation in which confers resistance to imidazolinones. Residues Y47 to G50 are also in contact with TPP. This loop is adjacent to P184-Q189, another turn, which connects the last strand of the β -sheet of domain 1 with a β -strand that connects with domain 2. Within the pocket, near its entrance, is a long region of domain 1 that interacts with a

complementary stretch of domain 2. Residues 125-129 and 133-137 of domain 1 and residues 304-313 of domain 2 are at the surface of the pocket. A turn consisting of T96-G100 is between loop 125-129 and TPP. A further stretch of domain 3 and two regions of domain 2 that line the binding pocket are at the opposite corner of the pocket. Residues 572, 575, 582, and 583 of domain 3 define the pocket surface on one side. The remaining part of the interior of the pocket's surface is defined by FAD and by a loop, L278-G282, that contacts the isoalloxazine ring of FAD.

The structural models of the AHAS protein can also be used for the rational design of herbicides or AHAS inhibitors.

2. Modelling of Herbicides Into Binding Sites

Imazethapyr, the active imidazolinone in PURSUIT®, was positioned into its proposed binding site using interactive molecular graphics (Figure 4) and the software described above (Figure 4). K185 was chosen as an "anchor" to interact with the charge of the carboxyl group. The imidazolinone's NH-CO unit was placed to form hydrogen bonds to G50 and A51. This positioned the methyl substitute of imazethapyr close to V500 on the backbone of the small α -helix. The isopropyl group is possibly bound by hydrophobic residues of the amino acids in the region of residues 125-135 that contribute to the inner surface of the pocket. The pyridine ring is most probably "sandwiched" between A134 or F135, F507 and W503. W503 also interacts with the imidazolinone ring system.

In a similar fashion, the sulfonylurea herbicides were modelled into a site that partially overlapped the described imidazolinone binding site. Overlap of sulfonylurea and imidazolinone binding sites was consistent with competition binding experiments and with established mutant data, which show that the same mutation in maize, W503L, can confer resistance to both herbicides. In these models, most of the known mutation sites that confer sulfonylurea herbicide resistance, i.e. G50, A51, K185, V500, W503, F507, are in close contact to the bound herbicides. P126 and A51 are required for keeping the K185 side chain in place by generating a

hydrophobic pore. S582, a site for specific imidazolinone resistance, is distant from the binding region and is located in the region where the homology is so poor that a change in the fold is expected. The FAD binding site apparently has low
5 homology between AHAS and POX in this region; S582 is a residue that confers resistance in maize, and that S582 and its adjacent residues are in close contact to the active site pocket. It is proposed that FAD and the loop region encompassing residues 278 to 285 move slightly away from the
10 third domain, (downward in Figure 4) and that a loop that contains S582 folds into the space between the helix at positions 499 to 507 and the loop at positions 278 to 285. D305, another known resistance site, is close to FAD and modulates the interaction between domains 1 and 2. M280 may
15 either be involved in positioning of the helix at positions 498 to 507 or directly in inhibitor binding. M280 and D305 could also be directly involved in inhibitor binding if domains 1 and 2 move slightly closer to each other.

3. Selection of Mutations

20 Specific amino acid residues are pinpointed as sites for the introduction of mutations into the primary sequence of AHAS. These amino acids are selected based upon their position in that if that amino acid residue position is modified, there will be a resultant alteration (i.e. decline) in the affinity
25 of an herbicide for the binding pocket. It is not necessary that the mutation position reside in the binding pocket as amino acid residues outside the pocket itself can alter the pocket charge or configuration. The selection of target sites for mutation is achieved using molecular models as described
30 above. For example according to the model above, arginine at position 128 (designated R128 in Figure 1 using the single-letter code for amino acids) is located near the entrance to the substrate- and herbicide-binding pocket and has a large degree of conformational freedom that may allow it to
35 participate in transport of charged herbicides into the binding pocket. Therefore, this residue is substituted by alanine to remove both its charge and its long hydrophobic side chain. (The resulting mutation is designated R128A).

The mutations may comprise simple substitutions, which replace the wild-type sequence with any other amino acid. Alternatively, the mutations may comprise deletions or additions of one or more amino acids, preferably up to 5, at a given site. The added sequence may comprise an amino acid sequence known to exist in another protein, or may comprise a completely synthetic sequence. Furthermore, more than one mutation and/or more than one type of mutation may be introduced into a single polypeptide.

10 4. Site-Directed Mutagenesis

The DNA encoding AHAS can be manipulated so as to introduce the desired mutations. Mutagenesis is carried out using methods that are standard in the art, as described in, for example, Higuchi, R., *Recombinant PCR*, In M.A. Innis, et al., eds, *PCR Protocols: A Guide to Methods and Applications*, Academic Press, pp. 177-183, 1990.

 5. Expression and Purification of Variants

The mutated or variant AHAS sequence is cloned into a DNA expression vector (see, e.g., Example 3) and is expressed in a suitable cell such as, for example, *E. coli*. Preferably, the DNA encoding AHAS is linked to a transcription regulatory element, and the variant AHAS is expressed as part of a fusion protein, for example, glutathione-S-transferase, to facilitate purification (see Example 3 below). The variant AHAS is then purified using affinity chromatography or any other suitable method known in the art. "Purification" of an AHAS polypeptide refers to the isolation of the AHAS polypeptide in a form that allows its enzymatic activity to be measured without interference by other components of the cell in which the polypeptide is expressed.

 6. Assaying of Enzymatic Properties

The purified variant AHAS may be assayed for one or more of the following three properties:

(a) specific or catalytic activity for conversion of pyruvate to acetolactate (expressed as units/mg pure AHAS, wherein a unit of activity is defined as 1 μ mole acetolactate produced/hour), or for condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate (expressed as

units/mg pure AHAS, wherein a unit of activity is defined as 1 μ mole acetohydroxybutyrate produced/hr.;

(b) level of inhibition by herbicide, such as, for example, imidazolinone (expressed as IC_{50} , the concentration at which 50% of the activity of the enzyme is inhibited); and

(c) selectivity of resistance to the selected herbicide vs. other herbicides. The selectivity index is defined as the fold resistance of the mutant to imidazolinones relative to the wild-type enzyme, divided by the fold resistance of the same mutant to other herbicides also relative to the wild-type). Fold resistance to an herbicide relative to the wild-type enzyme is expressed as the IC_{50} of variant, divided by the IC_{50} of the wild type. The selectivity index (S.I.) is thus represented by the following equation:

$$S.I. = \frac{IC_{50} \text{ of variant for herb.A} / IC_{50} \text{ of wild type for herb.A}}{IC_{50} \text{ of variant for herb.B} / IC_{50} \text{ of wild type for herb.B.}}$$

Suitable assay systems for making these determinations include, but are not limited to, those described in detail in Example 4 below.

7.a. Evaluation of Suitable Variants

The enzymatic properties of variant AHAS polypeptides are compared to the wild-type AHAS. Preferably, a given mutation results in an AHAS variant polypeptide that retains *in vitro* enzymatic activity towards pyruvate or pyruvate and 2-ketobutyrate, i.e., the conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate (and thus is expected to be biologically active *in vivo*), while exhibiting catalytic activity that is relatively more resistant to the selected herbicide(s) than is wild-type AHAS. Preferably, the variant AHAS exhibits:

(i) in the absence of the at least one herbicide,

(a) catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(b) catalytic activity in combination with any herbicide resistant AHAS variant protein also

expressed in the cell, which may be the same as or different than the first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein the cell requires AHAS
5 activity for viability; and

(ii) catalytic activity that is more resistant to the at least one herbicide than is wild type AHAS;

and that is relatively more resistant to the
10 herbicide(s) than is wild-type AHAS.

Therefore, any one specific AHAS variant protein need not have the total catalytic activity necessary to maintain the viability of the cell, but must have some catalytic activity in an amount, alone or in combination with the catalytic
15 activity of additional copies of the same AHAS variant and/or the catalytic activity of other AHAS variant protein(s), sufficient to maintain the viability of a cell that requires AHAS activity for viability. For example, catalytic activity may be increased to minimum acceptable levels by introducing
20 multiple copies of a variant encoding gene into the cell or by introducing the gene which further includes a relatively strong promoter to enhance the production of the variant.

More resistant means that the catalytic activity of the variant is diminished by the herbicide(s), if at all, to
25 a lesser degree than wild-type AHAS catalytic activity is diminished by the herbicide(s). Preferred more resistant variant AHAS retains sufficient catalytic to maintain the viability of a cell, plant, or organism wherein at the same concentration of the same herbicide(s), wild-type AHAS would
30 not retain sufficient catalytic activity to maintain the viability of the cell, plant, or organism.

Preferably the catalytic activity in the absence of herbicide(s) is at least about 5% and, most preferably, is more than about 20% of the catalytic activity of the wild-type AHAS
35 in the absence of herbicide(s). Most preferred AHAS variants are more resistant to imidazolinone herbicides than to other herbicides such as sulfonylurea-based herbicides, though in some applications selectivity is neither needed nor preferred.

In the case of imidazolinone-resistant variant AHAS, it is preferred that the AHAS variant protein has

(i) catalytic activity in the absence of said herbicide of more than about 20% of the catalytic activity of said wild-type AHAS;

(ii) catalytic activity that is relatively more resistant to presence of imidazolinone herbicides compared to wild type AHAS; and

(iii) catalytic activity that is relatively more sensitive to the presence of sulfonylurea herbicides compared to imidazolinone herbicides. Most preferred herbicide-resistant AHAS variants exhibit a minimum specific activity of about 20 units/mg, minimal or no inhibition by imidazolinone, and a selectivity index ranging from about 1.3 to about 3000 relative to other herbicides.

Without wishing to be bound by theory, it is believed that systematic and iterative application of this method to wild type or other target AHAS protein will result in the production of AHAS variants having the desired properties of high enzymatic activity as explained above and resistance to one or more classes of herbicides. For example, mutation of a wild-type AHAS sequence at a particular position to a given amino acid may result in a mutant that exhibits a high degree of herbicide resistance but a significant loss of enzymatic activity towards pyruvate or pyruvate and 2-ketobutyrate. In a second application of the above method, the starting or target AHAS polypeptide would then be this variant (in place of the wild-type AHAS). Rational design then involves substituting other amino acids at the originally mutated position and/or adding or deleting amino acids at selected points or ranges in the expectation of retaining herbicide resistance but also maintaining a higher level of enzymatic activity.

The structure-based rational design of herbicide resistant AHAS proteins offers many advantages over conventional approaches that rely on random mutagenesis and selection. For example, when substitution of a particular amino acid with another requires substitution of more than one

nucleotide within the codon, the likelihood of this occurring randomly is so low as to be impractical. By contrast, even double or triple changes in nucleotide sequence within a codon can be easily implemented when suggested by a rational design approach. For example, one rationally designed mutation to confer selective imidazolinone resistance requires a change from arginine to glutamate. Arginine is encoded by CGT, CGC, CGA, CGG, AGA, AGG, while glutamate is encoded by GAA and GAG. Since none of the arginine codons begins with GA, this mutation would require a double substitution of adjacent nucleotides which would occur so rarely using random mutagenesis as to be unpredictable and unrepeatable with any certainty of success. Although mutation frequency can be increased during random mutagenesis, alterations in nucleotide sequence would have an equal probability of occurring throughout the AHAS gene, in the absence of prior site-direction of the mutations. This increases the chance of obtaining an irrelevant mutation that interferes with enzymatic activity. Similarly, it would be rare, using random mutagenesis, to find a multiple amino acid substitution, deletion, or substitution/deletion mutation that confers herbicide resistance while maintaining catalytic activity. Deletion mutations that confer herbicide resistance would also be unlikely using a random mutagenesis approach. Deletions would need to be limited to small regions and would have to occur in triplets so as to retain the AHAS reading frame in order to retain enzymatic activity.

However, with a rational structure-based approach, double amino acid substitution and/or deletion mutations are relatively easily achieved and precisely targeted. Furthermore, different mutagens used in random mutagenesis create specific types of mutations. For example, sodium azide creates point substitution mutations in plants, while radiation tends to create deletions. Accordingly, two mutagenesis protocols would have to be employed to obtain a multiple combination substitution/deletion.

Finally, the present structure-based method for rational design of herbicide-resistant AHAS variants allows for iterative improvement of herbicide resistance mutations, a step

that is not facilitated by random mutagenesis. Identification of a mutation site for herbicide resistance by random mutagenesis may offer little, if any, predictive value for guiding further improvements in the characteristics of the mutant. The present structure-based approach, on the other hand, allows improvements to be implemented based on the position, environment, and function of the amino acid position in the structural model.

The iterative improvement method also allows the independent manipulation of three important properties of AHAS: level of resistance, selectivity of resistance, and catalytic efficiency. For example, compensatory mutations can be designed in a predictive manner. If a particular mutation has a deleterious effect on the activity of an enzyme, a second compensatory mutation may be used to restore activity. For example, a change in the net charge within a domain when a charged residue is introduced or lost due to a mutation can be compensated by introducing a second mutation. Prediction of the position and type of residue(s) to introduce, delete, or substitute at the second site in order to restore enzymatic activity requires a knowledge of structure-function relationships derived from a model such as that described herein.

7.b. Design of Non-Peptide Herbicides or AHAS Inhibitors

A chemical entity that alters and may fit into the active site of the target protein or bind in any position where it could inhibit activity may be designed by methods known in the art, such as, for example, computer design programs that assist in the design of compounds that specifically interact with a receptor site.

An example of such a program is LUDI (Biosym Technologies - San Diego, CA) (see also, Lam, et al., *Science* **263**:380, 1994; Thompson, et al., *J. Med. Chem.*, **37**:3100, 1994).

The binding pocket and particularly the amino acid residues that have been identified as being involved as inhibitor binding can be used as anchor points for inhibitor design.

The design of site-specific herbicides is advantageous in the control of weed species that may spontaneously develop herbicide resistance in the field, particularly due to mutations in the AHAS gene.

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Herbicide-Resistant AHAS Variants: DNA, Vectors, and Polypeptides

The present invention also encompasses isolated DNA molecules encoding variant herbicide-resistant AHAS polypeptides. Genes encoding AHAS polypeptides according to the present invention may be derived from any species and preferably a plant species, and mutations conferring herbicide resistance may be introduced at equivalent positions within any of these AHAS genes. The equivalence of a given codon position in different AHAS genes is a function of both the conservation of primary amino acid sequence and its protein and the retention of similar three-dimensional structure. For example, Figure 5 illustrates the high degree of sequence homology between AHAS polypeptides derived from different plant species. These AHAS polypeptides exhibit at least about 60 to about 70% overall homology. Without wishing to be bound by theory, it is believed that in regions of the polypeptide having a highly conserved sequence, the polypeptide chain conformation will also be preserved. Thus, it is possible to use an AHAS-encoding sequence from one species for molecular modelling, to introduce mutations predictively into an AHAS gene from a second species for initial testing and iterative improvement, and finally, to introduce the optimized mutations into AHAS derived from yet a third plant species for expression in a transgenic plant.

In one series of embodiment, these AHAS DNAs encode variants of an AHAS polypeptide and preferably of the maize AHAS polypeptide of Figure 1 in which the polypeptide is modified by substitution at or deletion preceding or following one or more of Figure 1 amino acid residues P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281,

G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, 5 L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional 10 equivalents of any of the foregoing; insertions or deletions between Figure 1 Q124 and H150 or functional equivalents thereof; insertions or deletions between Figure 1 G300 and D324 or functional equivalents thereof; and any combination of any of the foregoing thereof.

15 The mutations, whether introduced into the polypeptide of Figure 1 or at equivalent positions in another plant AHAS gene, may comprise alterations in DNA sequence that result in a simple substitution of any one or more other amino acids or deletions of up to 5 amino acid residues proceeding 20 or up to 5 amino acids residues following any of the residence listed above. Suitable amino acid substituents include, but are not limited to, naturally occurring amino acids.

Alternatively, the mutations may comprise alterations in DNA sequence such that one or more amino acids are added or 25 deleted in frame at the above positions. Preferably, additions comprise about 3 to about 30 nucleotides, and deletions comprise about 3 to about 30 nucleotides. Furthermore, a single mutant polypeptide may contain more than one similar or different mutation.

30 The present invention encompasses DNA and corresponding RNA sequences, as well as sense and antisense sequences. Nucleic acid sequences encoding AHAS polypeptides may be flanked by natural AHAS regulatory sequences, or may be associated with heterologous sequences, including promoters, 35 enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. Furthermore, the nucleic acids can be modified to alter stability, solubility, binding affinity and specificity. For

example, variant AHAS-encoding sequences can be selectively methylated. The nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

The invention also provides vectors comprising nucleic acids encoding AHAS variants. A large number of vectors, including plasmid and fungal vectors, have been described for expression in a variety of eukaryotic and prokaryotic hosts. Advantageously, vectors may also include a promoter operably linked to the AHAS encoding portion. The encoded AHAS may be expressed by using any suitable vectors and host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Examples of suitable vectors include without limitation pBIN-based vectors, pBluescript vectors, and pGEM vectors.

The present invention also encompasses both variant herbicide-resistant AHAS polypeptides or peptide fragments thereof. As explained above, the variant AHAS polypeptides may be derived from the maize polypeptide shown in Figure 1 or from any plant or microbial AHAS polypeptide, preferably plant AHAS polypeptide. The polypeptides may be further modified by, for example, phosphorylation, sulfation, acylation, glycosylation, or other protein modifications. The polypeptides may be isolated from plants, or from heterologous organisms or cells (including, but not limited to, bacteria, yeast, insect, plant, and mammalian cells) into which the gene encoding a variant AHAS polypeptide has been introduced and expressed. Furthermore, AHAS polypeptides may be modified with a label capable of providing a detectable signal, either directly or indirectly, including radioisotopes, fluorescent compounds, and the like.

35 Chemical-resistant Plants and Plants Containing Variant AHAS Genes

The present invention encompasses transgenic cells, including, but not limited to seeds, organisms, and plants into

which genes encoding herbicide-resistant AHAS variants have been introduced. Non-limiting examples of suitable recipient plants are listed in Table 1 below:

TABLE 1
RECIPIENT PLANTS

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<u>COMMON NAME</u>	<u>FAMILY</u>	<u>LATIN NAME</u>
Maize	Gramineae	Zea mays
Maize, Dent	Gramineae	Zea mays dentiformis
Maize, Flint	Gramineae	Zea mays vulgaris
Maize, Pop	Gramineae	Zea mays microsperma
Maize, Soft	Gramineae	Zea mays amylacea
Maize, Sweet	Gramineae	Zea mays amyleasaccharata
Maize, Sweet	Gramineae	Zea mays saccharate
Maize, Waxy	Gramineae	Zea mays ceratina
Wheat, Dinkel	Pooideae	Triticum spelta
Wheat, Durum	Pooideae	Triticum durum
Wheat, English	Pooideae	Triticum turgidum
Wheat, Large Spelt	Pooideae	Triticum spelta
Wheat, Polish	Pooideae	Triticum polonium
Wheat, Poulard	Pooideae	Triticum turgidum
Wheat, Singlegrained	Pooideae	Triticum monococcum
Wheat, Small Spelt	Pooideae	Triticum monococcum
Wheat, Soft	Pooideae	Triticum aestivum
Rice	Gramineae	Oryza sativa
Rice, American Wild	Gramineae	Zizania aquatica
Rice, Australian	Gramineae	Oryza australiensis
Rice, Indian	Gramineae	Zizania aquatica
Rice, Red	Gramineae	Oryza glaberrima
Rice, Tuscarora	Gramineae	Zizania aquatica
Rice, West African	Gramineae	Oryza glaberrima
Barley	Pooideae	Hordeum vulgare
Barley, Abyssinian Intermediate, also Irregular	Pooideae	Hordeum irregulare
Barley, Ancestral Tworow	Pooideae	Hordeum spontaneum
Barley, Beardless	Pooideae	Hordeum trifurcatum
Barley, Egyptian	Pooideae	Hordeum trifurcatum
Barley, fourrowed	Pooideae	Hordeum vulgare polystichon

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	<u>COMMON NAME</u>	<u>FAMILY</u>	<u>LATIN NAME</u>
	Barley, sixrowed	Pooideae	Hordeum vulgare hexastichon
	Barley, Tworowed	Pooideae	Hordeum distichon
5	Cotton, Abroma	Dicotyledoneae	Abroma augusta
	Cotton, American Upland	Malvaceae	Gossypium hirsutum
	Cotton, Asiatic Tree, also Indian Tree	Malvaceae	Gossypium arboreum
10	Cotton, Brazilian, also, Kidney, and, Pernambuco	Malvaceae	Gossypium barbadense brasiliense
	Cotton, Levant	Malvaceae	Gossypium herbaceum
15	Cotton, Long Silk, also Long Staple, Sea Island	Malvaceae	Gossypium barbadense
20	Cotton, Mexican, also Short Staple	Malvaceae	Gossypium hirsutum
	Soybean, Soya	Leguminosae	Glycine max
25	Sugar beet	Chenopodiaceae	Beta vulgaris altissima
	Sugar cane	Woody-plant	Arenga pinnata
	Tomato	Solanaceae	Lycopersicon esculentum
30	Tomato, Cherry	Solanaceae	Lycopersicon esculentum cerasiforme
	Tomato, Common	Solanaceae	Lycopersicon esculentum commune
	Tomato, Currant	Solanaceae	Lycopersicon pimpinellifolium
	Tomato, Husk	Solanaceae	Physalis ixocarpa
35	Tomato, Hyenas	Solanaceae	Solanum incanum
	Tomato, Pear	Solanaceae	Lycopersicon esculentum pyriforme
	Tomato, Tree	Solanaceae	Cyphomandra betacea
	Potato	Solanaceae	Solanum tuberosum
40			
	Potato, Spanish, Sweet potato	Convolvulaceae	Ipomoea batatas
	Rye, Common	Pooideae	Secale cereale
45	Rye, Mountain	Pooideae	Secale montanum

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	<u>COMMON NAME</u>	<u>FAMILY</u>	<u>LATIN NAME</u>
	Pepper, Bell	Solanaceae	Capsicum annuum grossum
	Pepper, Bird, also Cayenne, Guinea	Solanaceae	Capsicum annuum minimum
5	Pepper, Bonnet	Solanaceae	Capsicum sinense
	Pepper, Bullnose, also Sweet	Solanaceae	Capsicum annuum grossum
	Pepper, Cherry	Solanaceae	Capsicum annuum cerasiforme
10	Pepper, Cluster, also Red Cluster	Solanaceae	Capsicum annuum fasciculatum
	Pepper, Cone	Solanaceae	Capsicum annuum conoides
	Pepper, Goat, also Spur	Solanaceae	Capsicum frutescens
15	Pepper, Long	Solanaceae	Capsicum frutescens longum
	Pepper, Oranamental Red, also Wrinkled	Solanaceae	Capsicum annuum abbreviatum
20	Pepper, Tabasco Red	Solanaceae	Capsicum annuum conoides
	Lettuce, Garden	Compositae	Lactuca sativa
25	Lettuce, Asparagus, also Celery	Compositae	Lactuca sativa asparagina
	Lettuce, Blue	Compositae	Lactuca perennis
	Lettuce, Blue, also Chicory	Compositae	Lactuca pulchella
30	Lettuce, Cabbage, also Head	Compositae	Lactuca sativa capitata
	Lettuce, Cos, also Longleaf, Romaine	Compositae	Lactuca sativa longifolia
35	Lettuce, Crinkle, also Curled, Cutting, Leaf	Compositae	Lactuca sativa crispa
40	Celery	Umbelliferae	Apium graveolens dulce
	Celery, Blanching, also Garden	Umbelliferae	Apium graveolens dulce
45	Celery, Root, also Turniprooted	Umbelliferae	Apium graveolens rapaceum
	Eggplant, Garden	Solanaceae	Solanum melongena

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	<u>COMMON NAME</u>	<u>FAMILY</u>	<u>LATIN NAME</u>
	Sorghum	Sorghum	All crop species
	Alfalfa	Leguminosae	Medicago sativum
5	Carrot	Umbelliferae	Daucus carota sativa
	Bean, Climbing	Leguminosae	Phaseolus vulgaris vulgaris
	Bean, Sprouts	Leguminosae	Phaseolus aureus
10	Bean, Brazilian Broad	Leguminosae	Canavalia ensiformis
	Bean, Broad	Leguminosae	Vicia faba
	Bean, Common, also French, White, Kidney	Leguminosae	Phaseolus vulgaris
15	Bean, Egyptian	Leguminosae	Dolichos lablab
	Bean, Long, also Yardlong	Leguminosae	Vigna sesquipedalis
	Bean, Winged	Leguminosae	Psophocarpus tetragonolobus
20	Oat, also Common, Side, Tree	Avena	Sativa
	Oat, Black, also Bristle, Lopsided	Avena	Strigosa
25	Oat, Bristle	Avena	
	Pea, also Garden, Green, Shelling	Leguminosae	Pisum, sativum sativum
30	Pea, Blackeyed	Leguminosae	Vigna sinensis
	Pea, Edible Podded	Leguminosae	Pisum sativum axiphium
	Pea, Grey	Leguminosae	Pisum sativum speciosum
35	Pea, Winged	Leguminosae	Tetragonolobus purpureus
	Pea, Wrinkled	Leguminosae	Pisum sativum medullare
	Sunflower	Compositae	Helianthus annuus
40	Squash, Autumn, Winter	Dicotyledoneae	Cucurbita maxima
	Squash, Bush, also Summer	Dicotyledoneae	Cucurbita pepo melopepo
	Squash, Turban	Dicotyledoneae	Cucurbita maxima turbaniformis
45	Cucumber	Dicotyledoneae	Cucumis sativus

	<u>COMMON NAME</u>	<u>FAMILY</u>	<u>LATIN NAME</u>
	Cucumber, African, also Bitter		Momordica charantia
5	Cucumber, Squirting, also Wild		Ecballium elaterium
	Cucumber, Wild		Cucumis anguria
10	Poplar, California	Woody-Plant	Populus trichocarpa
	Poplar, European Black		Populus nigra
	Poplar, Gray		Populus canescens
	Poplar, Lombardy		Populus italica
15	Poplar, Silverleaf, also White		Populus alba
	Poplar, Western Balsam		Populus trichocarpa
20			
	Tobacco	Solanaceae	Nicotiana
	Arabidopsis Thaliana	Cruciferae	Arabidopsis thaliana
25			
	Turfgrass	Lolium	
	Turfgrass	Agrostis	
		Other families of turfgrass	
30	Clover	Leguminosae	

Expression of the variant AHAS polypeptides in transgenic plants confers a high level of resistance to herbicides including, but not limited to, imidazolinone herbicides such as, for example, imazethapyr (PURSUIT®), allowing the use of these herbicides during cultivation of the transgenic plants.

Methods for the introduction of foreign genes into plants are known in the art. Non-limiting examples of such methods include *Agrobacterium* infection, particle bombardment, polyethylene glycol (PEG) treatment of protoplasts, electroporation of protoplasts, microinjection, macroinjection, tiller injection, pollen tube pathway, dry seed imbibition, laser perforation, and electrophoresis. These methods are described in, for example, B. Jenes et al., and S.W. Ritchie

et al. In *Transgenic Plants, Vol. 1, Engineering and Utilization*, ed. S.-D. Kung, R. Wu, Academic Press, Inc., Harcourt Brace Jovanovich 1993; and L. Mannonen et al., *Critical Reviews in Biotechnology*, 14:287-310, 1994.

5 In a preferred embodiment, the DNA encoding a variant AHAS is cloned into a DNA vector containing an antibiotic resistance marker gene, and the recombinant AHAS DNA-containing plasmid is introduced into *Agrobacterium tumefaciens* containing a Ti plasmid. This "binary vector system" is described in, for
10 example, U.S. Patent No. 4, 490,838, and in An et al., *Plant Mol.Biol.Manual A3*:1-19 (1988). The transformed *Agrobacterium* is then co-cultivated with leaf disks from the recipient plant to allow infection and transformation of plant cells. Transformed plant cells are then cultivated in regeneration
15 medium, which promotes the formation of shoots, first in the presence of the appropriate antibiotic to select for transformed cells, then in the presence of herbicide. In plant cells successfully transformed with DNA encoding herbicide-resistant AHAS, shoot formation occurs even in the presence of
20 levels of herbicide that inhibit shoot formation from non-transformed cells. After confirming the presence of variant AHAS DNA using, for example, polymerase chain reaction (PCR) analysis, transformed plants are tested for their ability to withstand herbicide spraying and for their capabilities for
25 seed germination and root initiation and proliferation in the presence of herbicide.

Other Applications

The methods and compositions of the present invention
30 can be used in the structure-based rational design of herbicide-resistant AHAS variants, which can be incorporated into plants to confer selective herbicide resistance on the plants. Intermediate variants of AHAS (for example, variants that exhibit sub-optimal specific activity but high resistance
35 and selectivity, or the converse) are useful as templates for the design of second-generation AHAS variants that retain adequate specific activity and high resistance and selectivity.

Herbicide resistant AHAS genes can be transformed into crop species in single or multiple copies to confer herbicide resistance. Genetic engineering of crop species with reduced sensitivity to herbicides can:

5 (1) Increase the spectrum and flexibility of application of specific effective and environmentally benign herbicides such as imidazolinone herbicides;

(2) Enhance the commercial value of these herbicides;

10 (3) Reduce weed pressure in crop fields by effective use of herbicides on herbicide resistant crop species and a corresponding increase in harvest yields;

(4) Increase sales of seed for herbicide-resistant plants;

15 (5) Increase resistance to crop damage from carry-over of herbicides applied in a previous planting;

(6) Decrease susceptibility to changes in herbicide characteristics due to adverse climate conditions; and

20 (7) Increase tolerance to unevenly or mis-applied herbicides.

For example, transgenic AHAS variant protein containing plants can be cultivated. The crop can be treated with a weed controlling effective amount of the herbicide to which the AHAS variant transgenic plant is resistant, resulting
25 in weed control in the crop without detrimentally affecting the cultivated crop.

The DNA vectors described above that encode herbicide-resistant AHAS variants can be further utilized so that expression of the AHAS variant provides a selectable
30 marker for transformation of cells by the vector. The intended recipient cells may be in culture or *in situ*, and the AHAS variant genes may be used alone or in combination with other selectable markers. The only requirement is that the recipient cell is sensitive to the cytotoxic effects of the cognate
35 herbicide. This embodiment takes advantage of the relative low cost and lack of toxicity of, for example, imidazolinone-based herbicides, and may be applied in any system that requires DNA-mediated transformation.

Exemplification with respect to Preferred Embodiments

The following examples are intended to illustrate the present invention without limitation.

5 Example 1: Design of herbicide-resistant AHAS variants

Residues located close to the proposed herbicide binding site of the model described in detail above were selected for mutagenesis in order to design an active AHAS polypeptide with decreased herbicide binding capacity. Each
10 site at the surface of the pocket was considered in terms of potential interactions with other residues in the pocket, as well as with cofactors and herbicides. For example, addition of positively charged residue(s) is expected to interfere with the charge distribution within the binding site, resulting in
15 a loss in affinity of binding of a negatively-charged herbicide.

Three residues were identified as most useful targets for mutagenesis:

(1) F135 was believed to interact with both the
20 isoalloxazine ring of FAD and with the aromatic group of the herbicides. In accordance with the strategy of introducing more charged residues into the binding pocket, this residue was changed to arginine.

(2) M53 contacts helix 498-507. This helix contains
25 known herbicide resistance mutation sites and is also implicated in TPP binding. Furthermore, substitution of glutamic acid at position 53 was believed to favor an interaction with K185, reducing the affinity of K185 for the carboxylate group of imazethapyr.

(3) R128 is located near the entrance to the pocket,
30 where it was believed to be involved in the initial transport of charged herbicides into the binding pocket. This residue was changed to alanine to remove both its charge and its long hydrophobic side chain.

Example 2: Site-directed mutagenesis of AHAS to produce herbicide-resistant variants

The *Arabidopsis* AHAS gene was inserted in-frame to the 3' end of the coding region of the glutathione S-transferase gene in the pGEX-2T vector (Pharmacia). Construction of the vector in this manner maintained the six amino acid thrombin recognition sequence at the junction of the expressed glutathione-S-transferase (GST)/AHAS fusion protein. Thrombin digestion of the expressed fusion protein results in an AHAS protein with an N-terminal starting position at the end of the transit peptide at a putative transit peptide processing site, with a residual N-terminal glycine derived from the thrombin recognition site. The final amino terminus of the cleaved AHAS protein consists of Gly-Ser-Ser-Ile-Ser. Site-directed mutations were introduced into the AHAS gene in this vector.

Site-directed mutations were constructed according to the PCR method of Higuchi (*Recombinant PCR*. In MA Innis, et al. *PCR Protocols: A Guide to Methods and Applications*, Academic Press, San Diego, pp. 177-183, 1990). Two PCR products, each of which overlap the mutation site, were amplified. The primers in the overlap region contained the mutation. The overlapping PCR amplified fragments were combined, denatured, and allowed to re-anneal together, producing two possible heteroduplex products with recessed 3'-ends. The recessed 3'-ends were extended by *Taq* DNA polymerase to produce a fragment that was the sum of the two overlapping PCR products containing the desired mutation. A subsequent re-amplification of this fragment with only the two "outside" primers resulted in the enrichment of the full-length product. The product containing the mutation was then re-introduced into the *Arabidopsis* AHAS gene in the pGEX-2T vector.

Example 3: Expression and Purification of AHAS Variants

A. Methods

E. Coli (DH5 α) cells transformed with the pGEX-2T vector containing either the maize wild type AHAS gene (vector designation pAC751), the *Arabidopsis* Ser653Asn mutant, or the

Arabidopsis Ile401Phe mutant were grown overnight in LB broth containing 50 $\mu\text{g/mL}$ ampicillin. The overnight culture of *E. coli* was diluted 1:10 in 1 L LB, 50 $\mu\text{g/mL}$ ampicillin, and 0.1% v/v antifoam A. The culture was incubated at 37°C with shaking
5 until the OD_{600} reached approximately 0.8. Isopropylthiogalactose (IPTG) was added to a final concentration of 1 mM and the culture was incubated for 3 more hours.

Cells were harvested by centrifugation at 8,670 xg
10 for 10 minutes in a JA-10 rotor and resuspended in 1/100th of the original culture volume in MTPBS (16 mM Na_2HPO_4 , 4 mM NaH_2PO_4 , 150 mM NaCl, pH 7.3). Triton X-100 and lysozyme were added to a final concentration of 1% v/v and 100 $\mu\text{g/mL}$, respectively. Cells were incubated at 30°C for 15 minutes
15 cooled to 4°C on ice, and were lysed by sonication for 10 seconds at level 7 with a Branson Sonifier Cell Disrupter equipped with a microtip probe. The cell free extract was centrifuged at 35,000 xg for 10 min. at 4°C. The supernatant was decanted and the centrifugation step was repeated.

Purification of expressed fusion proteins was
20 performed as modified from Smith and Johnson (Gene 67:31-40, 1988). The supernatant was warmed to room temperature and was passed through a 2 mL column of glutathione-agarose beads (sulfur linkage, Sigma) equilibrated in MTPBS. The column was
25 subsequently washed with MTPBS at room temperature until the A_{280} of eluant matched that of MTPBS. The fusion protein was then eluted using a solution containing 5 mM reduced glutathione in 50 mM Tris HCL, pH 8.0. The eluted fusion protein was treated with approximately 30 NIH units of thrombin
30 and dialyzed against 50 mM citrate pH 6.5 and 150 mM NaCl.

The fusion protein was digested overnight at room temperature. Digested samples were dialyzed against MTPBS and passed twice through a glutathione-agarose column equilibrated in MTPBS to remove the released glutathione transferase
35 protein. The protein fraction that did not bind to the column was collected and was concentrated by ultrafiltration on a YM10 filter (Amicon). The concentrated sample was loaded onto a 1.5 x 95 cm Sephacryl S-100 gel filtration column equilibrated in

gel filtration buffer (50 mM HEPES, 150 mM NaCl, pH 7.0). Two mL fractions were collected at a flow rate of 0.14 mL/min. Enzyme stability was tested by storage of the enzyme at 4°C in gel filtration buffer with the addition of 0.02% sodium azide and in the presence or absence of 2 mM thiamine pyrophosphate and 100 μ M flavin adenine dinucleotide (FAD).

B. Results

E. coli transformed with the pAC751 plasmid containing the wide-type AHAS gene fused downstream and in-frame with the GST gene expressed a 91 kD protein when induced with IPTG. The 91 kD protein exhibited the predicted molecular mass of a GST/AHAS fusion protein (the sum of 26 kD and 65 kD, respectively). When the cell free extract of DH5 α /pAC751 was passed through a glutathione-agarose affinity gel, washed, and eluted with free glutathione it yielded a preparation enriched in the 91 kD protein (Figure 6, lane C). The six amino acid thrombin recognition site engineered in the junction of GST and AHAS was successfully cleaved by thrombin (Figure 6, lane D). The cleaved fusion protein preparation consisted of the expected 26 kD GST protein and the 65 kD maize AHAS protein. Maize AHAS was purified to homogeneity by a second pass through the glutathione-agarose column to affinity subtract GST and subjected to a final Sephacryl S-100 gel filtration step to eliminate thrombin (Figure 6, lane E). The 65 kD protein is recognized on western blots by a monoclonal antibody raised against a maize AHAS peptide.

Purified wild type maize AHAS was analyzed by electrospray mass spectrometry and was determined to have a molecular mass of 64,996 daltons (data not shown). The predicted mass, as calculated from the deduced amino acid sequence of the gene inserted into the pGEX-2T vector, is 65,058. The 0.096% discrepancy between the empirically determined and predicted mass was within tuning variability of the mass spectrometer. The close proximity of the two mass determinations suggests that there were no misincorporated nucleotides during construction of the expression vector, nor any post-translational modifications to the protein that would cause gross changes in molecular mass. Moreover, the lack of

spurious peaks in the preparation of purified enzyme indicated that the sample was free of contamination.

Example 4: Enzymatic properties of AHAS variants

5 The enzymatic properties of wild-type and variant AHAS produced in *E. coli* were measured by a modification of the method of Singh et al. (*Anal. Biochem* 171:173-179, 1988) as follows:

10 A reaction mixture containing 1X AHAS assay buffer (50 mM HEPES pH 7.0, 100 mM pyruvate, 10 mM MgCl₂, 1 mM thiamine pyrophosphate (TPP), and 50 μM flavin adenine dinucleotide (FAD)) was obtained either by dilution of enzyme in 2X assay buffer or by addition of concentrated enzyme to 1X AHAS assay buffer. All assays containing imazethapyr and
15 associated controls contained a final concentration of 5% DMSO due to addition of imazethapyr to assay mixtures as a 50% DMSO solution. Assays were performed in a final volume of 250 μL at 37°C in microtiter plates. After allowing the reaction to proceed for 60 minutes, acetolactate accumulation was measured
20 colorimetrically as described by Singh et al., *Anal. Biochem* 171:173-179, 1988.

Maize AHAS expressed and purified from pAC751 as described in Example 3 above is active in the conversion of pyruvate to acetolactate. Full AHAS activity is dependent on
25 the presence of the cofactors FAD and TPP in the assay medium. No activity was detected when only FAD was added to the assay medium. The activity of the purified enzyme with TPP only, or with no cofactors, was less than 1% of the activity detected in the presence of both TPP and FAD. Normally, AHAS present
30 in crude plant extracts is very labile, particularly in the absence of substrate and cofactors. In contrast, the purified AHAS from the bacterial expression system showed no loss in catalytic activity when stored for one month at 4°C in 50 mM HEPES pH 7.0, 150 mM NaCl, 0.02% NaN₃ in the presence or
35 absence of FAD and TPP. Furthermore, no degradation products were visible from these stored preparations when resolved in SDS-PAGE gels.

The specific activities of wild-type AHAS and the M124E, R199A, and F206R variants are shown in Table 2 below. As determined from the alignment in Figure 5, the M124E mutation in *Arabidopsis* AHAS is the equivalent of the maize M53E mutation, the R199A mutation in *Arabidopsis* is the equivalent of the maize R128A mutation, and the F206R mutation in *Arabidopsis* is the equivalent of the maize F135R mutation. The mutations designed in the maize AHAS structural model were used to identify the equivalent amino acid in the dicot *Arabidopsis* AHAS gene and were incorporated and tested in the *Arabidopsis* AHAS gene. This translation and incorporation of rationally designed herbicide mutations into the dicot *Arabidopsis* AHAS gene can facilitate evaluation of herbicide resistance in plants of a dicot species.

TABLE 2**SPECIFIC ACTIVITY**

	Specific Activity	% Catalytic Activity as Compared to Wild Type
Wild-Type	99.8	100
Met124Glu	9.15	9.16
Arg199Ala	86.3	86.5
Phe206Arg	5.07	5.1

The R199A mutation maintains a high level of catalytic activity (Table 2) while exhibiting a significant level of resistance to imazethapyr (Figure 7). Notably, this variant retains complete sensitivity to sulfonylureas (Figure 8). Thus, this variant fulfills the criteria of high specific activity and selective herbicide resistance. By contrast, the M124E substitution resulted in almost complete resistance to imazethapyr (Figure 7) but also exhibited severely reduced catalytic activity (Table 2). Relative to imidazolinone resistance, this variant exhibits greater sensitivity to sulfonylurea (Figure 8), suggesting that this residue is a good candidate for creating a mutation that confers selective resistance. Substitution of an amino acid other than glutamic acid may help to maintain catalytic activity. The F206R

substitution yielded similar results to those observed with M124E variant, but lacked selectivity in resistance.

5 Example 5: Iterative Improvement of AHAS
 Herbicide-Resistant Variant
 Using a Rational Design
 Approach

 Changing residue 124 in AHAS from Met to Glu as described in Example 4 above conferred imidazolinone resistance
10 but also reduced enzymatic activity to 9.2% of the wild type value. The model of the maize AHAS structure described above suggested that Met53 (equivalent to the *Arabidopsis* Met124 residue) interacts with a series of hydrophobic residues on the face of an α -helix that is derived from a separate subunit but
15 are in close proximity to Met53. Thus, the hydrophobic interaction between Met53 and the residues on the helix may stabilize both subunit/subunit association and the conformation of the active site. It was believed that the substitution of the hydrophobic Met residue with a charged glutamate residue
20 most probably destabilizes the inter-subunit hydrophobic interaction and results in a loss of catalytic activity.

 Based on this structure/function analysis, the activity of the original *Arabidopsis* Met124Glu (equivalent to maize Met53Glu) mutant enzyme was then iteratively improved by
25 substituting a more hydrophobic amino acid (Ile) at this position. The hydrophobic nature of the Ile side chain resulted in restoration of activity to wild type levels (specific activity of 102, equivalent to 102% of the wild-type activity), but the greater bulk of the Ile side chain was still
30 able to maintain a significant level of imidazolinone resistance (Figure 9).

 By comparison, substitution of a histidine residue at this position resulting in an AHAS variant exhibiting a specific activity of 42.5, equivalent to 42.6% of the wild-type
35 activity. This mutant, nonetheless, exhibited a high degree of resistance to PURSUIT® (Figure 10).

Example 6: Iterative Improvement of AHAS
Herbicide-Resistant Variant
Using a Rational Design
Approach

5 Another example of iterative refinement using the
methods of the present invention involves the Arg128Ala
variant. The structural model of maize AHAS suggested that
the Arg128 residue, which resides at the lip of the herbicide
binding pocket, contributes to channeling charged substrates
10 and herbicides into the herbicide binding pocket and into the
active site. The Arg 128 residue is distant from the TPP
moiety, which binds the initial pyruvate molecule in the
reaction mechanism of AHAS, explaining why the substitution of
Arabidopsis AHAS Arg199 (the equivalent to maize Arg128) to
15 alanine had little effect on the catalytic activity of the
enzyme. The structural model further indicated that a more
radical change could be made at this position to raise the
level of resistance while maintaining high levels of catalytic
activity. On this basis, an iterative improvement of the
20 mutation was made to substitute the positively charge arginine
residue with a negatively charged glutamate residue. The
enzyme thus mutated had improved levels of resistance to
PURSUIT® while maintaining high levels of activity (specific
activity of 114, equivalent to 114% of the wild-type activity)
25 (Figure 11).

Example 7: Interchangeability of AHAS Derived From
Different Species in Structure-Based Rational
Design of Herbicide-Resistant AHAS Variants

30 A structural model of the three-dimensional structure
of AHAS is built with a monocot AHAS sequence such as that
derived from maize, as described above. To introduce mutations
into AHAS derived from a dicot species such as *Arabidopsis*, the
sequences of AHAS derived from the monocot and dicot species
35 are aligned using the GAP and PILEUP programs (Genetics
Computer Group, 575 Sequence Drive, Madison, WI 53711).
Equivalent positions are determined from the computer-generated
alignment. The mutations are then introduced into the dicot
AHAS gene as described above. Following expression of the
40 mutant AHAS protein in *E. coli* and assessment of its

biochemical properties (i.e., specific activity and resistance to herbicides), the mutant gene is introduced into a dicot plant by plant transformation methods as described above.

5 Example 8: Production of Herbicide-Resistant Plants by Transformation with Rationally Designed AHAS Genes

DNA Constructs:

10 Rationally designed AHAS variant genes contained within *E. coli* expression vectors were used as a source of DNA restriction fragments to replace the equivalent restriction fragment in a *Arabidopsis* AHAS gene. This gene is present in a 5.5 kb genomic DNA fragment which also contains the
15 *Arabidopsis* AHAS promoter, the *Arabidopsis* AHAS termination sequence and 5'- and 3'-flanking DNA. After DNA sequencing through the mutation sites was performed to confirm the presence of the proper mutation, the entire 5.5 kb fragment from each plasmid was inserted into a pBIN based plant
20 transformation vector (Mogen, Leiden, Netherlands). The plant transformation vector also contains the neomycin phosphotransferase II (nptII) kanamycin resistance gene driven by the 35S cauliflower mosaic virus promoter. The final vector construct is displayed in Figure 12. Vectors containing
25 *Arabidopsis* AHAS genes with Met124Ile, Met124His, and Arg199Glu mutations (corresponding to Met53Ile, Met53His, and Arg128Glu mutations in the maize AHAS sequence as shown in Figure 1) were labeled pJK002, pJK003, and pJK004, respectively.

Each of these vectors was transformed into
30 *Agrobacterium tumefaciens* strain LBA4404 (R&D Life Technologies, Gaithersburg, MD) using the transformation method described in An et al., *Plant Mol.Biol.Manual* **A3**:1-19 (1988).

Plant Transformation:

Leaf disc transformation of *Nicotiana tabacum* cv. Wisconsin 38 was performed as described by Horsch et al. (Science, **227**: 1229-1231, 1985) with slight modifications. Leaf discs were cut from plants grown under sterile conditions and co-cultivated upsidedown in Murashige Skoog media (Sigma Chemical Co., St. Louis, MO) for 2-3 days at 25°C in darkness

with *Agrobacterium tumefaciens* strains containing plasmids pJK002, pJK003, or pJK004. The discs were blotted dry and transferred to regeneration Murashige Skoog medium with B5 vitamins containing 1 mg/L benzyladenine and 0.1 mg/l 1-Naphthyl Acetic Acid, 100 mg/L kanamycin, and 500 mg/L cefotaxime (all obtained from Sigma).

Initially, transformants were selected by kanamycin resistance conferred by the nptII gene present in the transformation vector. Shoots derived from the leaf discs were excised and placed on fresh Murashige Skoog hormone free media containing cefotaxime and kanamycin.

In Vivo Herbicide Resistance

Kanamycin-resistant tobacco shoots were transferred to medium containing a 0.25 μ M imazethapyr. At this concentration of the imidazolinone herbicide, non-transformed tobacco shoots (containing endogenous wild-type AHAS) were not able to initiate root formation. By contrast, root initiation and growth were observed from tobacco shoots transformed with each of the mutant AHAS genes. Roots developed from shoots transformed with the Met124Ile and Arg199Glu mutant genes along with wild type are shown in Figure 1. Furthermore, plants transformed with the Met124Ile or Arg199Glu mutant genes were resistant to spraying with twice the field rate (100 g/ha) of imazethapyr (Figure 13). The patterns of root growth in transformed vs. non-transformed plants in the presence of herbicide, as well as the behavior after herbicide spraying suggest that expression of the rationally designed herbicide resistance genes confers herbicide resistance *in vivo*.

Detection of the Rationally Designed Genes in Herbicide Resistant Tobacco

Genomic DNA was isolated from the AHAS-transformed tobacco plants, and the presence of the *Arabidopsis* AHAS variant genes was verified by PCR analysis. Differences between the nucleotide sequences of the *Arabidopsis* AHAS gene and the two tobacco AHAS genes were exploited to design PCR primers that amplify only the *Arabidopsis* gene in a tobacco genomic DNA background. The rationally designed herbicide resistance genes were detected, as shown by amplification of

a DNA fragment of the proper size, in a majority of the herbicide resistant plants. No PCR signal was seen from non-transformed tobacco plants.

Segregation of Transformed AHAS Genes:

5 To monitor segregation of rationally designed AHAS genes in transformed plants, germination tests were performed. Seeds were placed in hormone-free Murashige-Skoog medium containing up to 2.5 μ M PURSUIT® and 100 μ M kanamycin. The seedlings that resulted were visually scored for resistance or
10 susceptibility to the herbicide.

Since tobacco plants are diploid, it would be expected that the progeny of self-pollinated plants should segregate 3:1 resistant:susceptible, reflecting the existence of 1 seedling homozygous for the resistant AHAS gene, 2
15 seedlings heterozygous for the resistant AHAS gene, and 1 seedling lacking a resistant AHAS gene.

The results indicate that resistant AHAS genes are segregating in the expected 3:1 ratio, supporting the conclusion that herbicide resistance is conferred by a single,
20 dominant copy of a rationally designed AHAS gene.

These results indicate that rational design of herbicide-resistant AHAS genes can be used to produce plants that exhibit herbicide resistant growth *in vivo*.

25 Example 9: Production of Plants Cross-Resistant to Different Herbicides by Transformation with Rationally Designed AHAS Genes

Tobacco plants transformed with rationally designed AHAS genes as described in Example 8 above were also tested
30 for cross-resistance to another herbicide, CL 299,263 (also known as imazamox). Germination tests were performed on seeds harvested from the primary transformants containing the Met124Ile, Met124His, and Arg199Glu *Arabidopsis* AHAS variant genes, in the absence or presence of 2.5 μ M CL 299,263 (Figure
35 15). This concentration of the herbicide causes severe stunting and bleaching of wild-type tobacco plants. Tobacco plants transformed with the Met124His AHAS gene showed the greatest level of resistance (Figure 15). Arg199Glu

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transformants showed an intermediate level of resistance, while Met124Ile showed little resistance (Figure 15).

Many variations of the present invention will suggest
5 themselves to those skilled in the art in light of the above
detailed description. Such obvious variations are within the
full intended scope of the appended claims.

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 78864-264 Seq 27-07-11 v1.txt).

The sequences in the sequence listing in electronic form are reproduced in the following table.

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Val	Glu	Ser 35	Leu	Glu	Arg	Cys	Gly 40	Val	Arg	Asp	Val	Phe	Ala 45	Tyr	Pro
Gly	Gly 50	Ala	Ser	Met	Glu	Ile 55	His	Gln	Ala	Leu	Thr 60	Arg	Ser	Pro	Val
Ile 65	Ala	Asn	His	Leu	Phe	Arg 70	His	Glu	Gln	Gly 75	Glu	Ala	Phe	Ala	Ala 80
Ser	Gly	Tyr	Ala	Arg 85	Ser	Ser	Gly	Arg	Val 90	Gly	Val	Cys	Ile 95	Ala	Thr
Ser	Gly	Pro	Gly 100	Ala	Thr	Asn	Leu	Val	Ser 105	Ala	Leu	Ala	Asp 110	Ala	Leu
Leu	Asp	Ser 115	Val	Pro	Met	Val	Ala	Ile	Thr 120	Gly	Gln	Val	Pro 125	Arg	Arg
Met	Ile 130	Gly	Thr	Asp	Ala	Phe	Gln	Glu	Thr 135	Pro	Ile	Val	Glu 140	Val	Thr

49b

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				165					170						175
Gly	Pro	Val	Leu	Val	Asp	Ile	Pro	Lys	Asp	Ile	Gln	Gln	Gln	Met	Ala
			180					185						190	
Val	Pro	Val	Trp	Asp	Lys	Pro	Met	Ser	Leu	Pro	Gly	Tyr	Ile	Ala	Arg
		195					200					205			
Leu	Pro	Lys	Pro	Pro	Ala	Thr	Glu	Leu	Leu	Glu	Gln	Val	Leu	Arg	Leu
	210					215					220				
Val	Gly	Glu	Ser	Arg	Arg	Pro	Val	Leu	Tyr	Val	Gly	Gly	Gly	Cys	Ala
225					230					235					240
Arg	Ser	Gly	Glu	Glu	Leu	Arg	Arg	Phe	Val	Glu	Leu	Thr	Gly	Ile	Pro
				245					250					255	
Val	Thr	Thr	Thr	Leu	Met	Gly	Leu	Gly	Asn	Phe	Pro	Ser	Asp	Asp	Pro
			260					265					270		
Leu	Ser	Leu	Arg	Met	Leu	Gly	Met	His	Gly	Thr	Val	Tyr	Ala	Asn	Tyr
		275					280					285			
Ala	Val	Asp	Lys	Ala	Asp	Leu	Leu	Leu	Ala	Leu	Gly	Val	Arg	Phe	Asp
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49c

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49d

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49e

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Arg	Ile	Glu	Asn	Leu	Pro	Val	Lys	Val	Phe	Val	Leu	Asn	Asn	Gln	His	485	490	495	
Leu	Gly	Met	Val	Val	Gln	Trp	Glu	Asp	Arg	Phe	Tyr	Lys	Ala	Asn	Arg	500	505	510	
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Thr	Lys	Lys	Asn	Glu	Val	Arg	Ala	Ala	Ile	Lys	Lys	Met	Leu	Glu	Thr	545	550	555	560
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49f

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 Val Glu Leu Thr Gly Ile Pro Val Thr Thr Thr Leu Met Gly Leu Gly
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 Ala Leu Gly Val Arg Phe Asp Asp Arg Val Thr Gly Lys Ile Glu Ala
 340 345 350

49g

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 Lys Ser Phe Asp Phe Gly Ser Trp Asn Asp Glu Leu Asp Gln Gln Lys
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 Arg Glu Phe Pro Leu Gly Tyr Lys Thr Ser Asn Glu Glu Ile Gln Pro
 420 425 430
 Gln Tyr Ala Ile Gln Val Leu Asp Glu Leu Thr Lys Gly Glu Ala Ile
 435 440 445
 Ile Gly Thr Gly Val Gly Gln His Gln Met Trp Ala Ala Gln Tyr Tyr
 450 455 460
 Thr Tyr Lys Arg Pro Arg Gln Trp Leu Ser Ser Ala Gly Leu Gly Ala
 465 470 475 480
 Met Gly Phe Gly Leu Pro Ala Ala Ala Gly Ala Ser Val Ala Asn Pro
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 Val Gln Glu Leu Ala Met Ile Arg Ile Glu Asn Leu Pro Val Lys Val
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 Phe Val Leu Asn Asn Gln His Leu Gly Met Val Val Gln Trp Glu Asp
 530 535 540
 Arg Phe Tyr Lys Ala Asn Arg Ala His Thr Tyr Leu Gly Asn Pro Glu
 545 550 555 560
 Asn Glu Ser Glu Ile Tyr Pro Asp Phe Val Thr Ile Ala Lys Gly Phe
 565 570 575
 Asn Ile Pro Ala Val Arg Val Thr Lys Lys Asn Glu Val Arg Ala Ala
 580 585 590
 Ile Lys Lys Met Leu Glu Thr Pro Gly Pro Tyr Leu Leu Asp Ile Ile
 595 600 605
 Val Pro His Gln Glu His Val Leu Pro Met Ile Pro Ser Gly Gly Ala
 610 615 620
 Phe Lys Asp Met Ile Leu Asp Gly Asp Gly Arg Thr Val Tyr
 625 630 635

<210> 5
 <211> 638
 <212> PRT
 <213> Zea mays

<400> 5
 Met Ala Thr Ala Ala Thr Ala Ala Ala Ala Leu Thr Gly Ala Thr Thr
 1 5 10 15
 Ala Thr Pro Lys Ser Arg Arg Arg Ala His His Leu Ala Thr Arg Arg
 20 25 30
 Ala Leu Ala Ala Pro Ile Arg Cys Ser Ala Leu Ser Arg Ala Thr Pro
 35 40 45
 Thr Ala Pro Pro Ala Thr Pro Leu Arg Pro Trp Gly Pro Asn Glu Pro
 50 55 60
 Arg Lys Gly Ser Asp Ile Leu Val Glu Ala Leu Glu Arg Cys Gly Val
 65 70 75 80
 Arg Asp Val Phe Ala Tyr Pro Gly Gly Ala Ser Met Glu Ile His Gln
 85 90 95

Ala	Leu	Thr	Arg	Ser	Pro	Val	Ile	Ala	Asn	His	Leu	Phe	Arg	His	Glu
			100					105					110		
Gln	Gly	Glu	Ala	Phe	Ala	Ala	Ser	Ala	Tyr	Ala	Arg	Ser	Ser	Gly	Arg
		115					120					125			
Val	Gly	Val	Cys	Ile	Ala	Thr	Ser	Gly	Pro	Gly	Ala	Thr	Asn	Leu	Val
	130					135					140				
Ser	Ala	Leu	Ala	Asp	Ala	Leu	Leu	Asp	Ser	Val	Pro	Met	Val	Ala	Ile
145					150					155					160
Thr	Gly	Gln	Val	Pro	Arg	Arg	Met	Ile	Gly	Thr	Asp	Ala	Phe	Gln	Glu
				165					170					175	
Thr	Pro	Ile	Val	Glu	Val	Thr	Arg	Ser	Ile	Thr	Lys	His	Asn	Tyr	Leu
			180					185					190		
Val	Leu	Asp	Val	Asp	Asp	Ile	Pro	Arg	Val	Val	Gln	Glu	Ala	Phe	Phe
		195					200					205			
Leu	Ala	Ser	Ser	Gly	Arg	Pro	Gly	Pro	Val	Leu	Val	Asp	Ile	Pro	Lys
	210					215					220				
Asp	Ile	Gln	Gln	Gln	Met	Ala	Val	Pro	Ala	Trp	Asp	Thr	Pro	Met	Ser
225					230					235					240
Leu	Pro	Gly	Tyr	Ile	Ala	Arg	Leu	Pro	Lys	Pro	Pro	Ala	Thr	Glu	Phe
				245					250					255	
Leu	Glu	Gln	Val	Leu	Arg	Leu	Val	Gly	Glu	Ser	Arg	Arg	Pro	Val	Leu
			260					265					270		
Tyr	Val	Gly	Gly	Gly	Cys	Ala	Ala	Ser	Gly	Glu	Glu	Leu	Cys	Arg	Phe
		275					280					285			
Val	Glu	Leu	Thr	Gly	Ile	Pro	Val	Thr	Thr	Thr	Leu	Met	Gly	Leu	Gly
	290					295					300				
Asn	Phe	Pro	Ser	Asp	Asp	Pro	Leu	Ser	Leu	Arg	Met	Leu	Gly	Met	His
305					310					315					320
Gly	Thr	Val	Tyr	Ala	Asn	Tyr	Ala	Val	Asp	Lys	Ala	Asp	Leu	Leu	Leu
				325					330					335	
Ala	Phe	Gly	Val	Arg	Phe	Asp	Asp	Arg	Val	Thr	Gly	Lys	Ile	Glu	Ala
			340					345					350		
Phe	Ala	Gly	Arg	Ala	Lys	Ile	Val	His	Ile	Asp	Ile	Asp	Pro	Ala	Glu
		355					360					365			
Ile	Gly	Lys	Asn	Lys	Gln	Pro	His	Val	Ser	Ile	Cys	Ala	Asp	Val	Lys
	370					375					380				
Leu	Ala	Leu	Gln	Gly	Met	Asn	Thr	Leu	Leu	Glu	Gly	Ser	Thr	Ser	Lys
385					390					395					400
Lys	Ser	Phe	Asp	Phe	Gly	Ser	Trp	His	Asp	Glu	Leu	Asp	Gln	Gln	Lys
			405						410					415	
Arg	Glu	Phe	Pro	Leu	Gly	Tyr	Lys	Ile	Phe	Asn	Glu	Glu	Ile	Gln	Pro
			420					425					430		
Gln	Tyr	Ala	Ile	Gln	Val	Leu	Asp	Glu	Leu	Thr	Lys	Gly	Glu	Ala	Ile
		435					440					445			
Ile	Ala	Thr													

49i

```

Arg Phe Tyr Lys Ala Asn Arg Ala His Thr Phe Leu Gly Asn Pro Glu
545          550          555          560
Asn Glu Ser Glu Ile Tyr Pro Asp Phe Val Ala Ile Ala Lys Gly Phe
          565          570          575
Asn Ile Pro Ala Val Arg Val Thr Lys Lys Ser Glu Val His Ala Ala
          580          585          590
Ile Lys Lys Met Leu Glu Ala Pro Gly Pro Tyr Leu Leu Asp Ile Ile
          595          600          605
Val Pro His Gln Glu His Val Leu Pro Met Ile Pro Ser Gly Gly Ala
          610          615          620
Phe Lys Asp Met Ile Leu Asp Gly Asp Gly Arg Thr Val Tyr
625          630          635

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```

<210> 6
<211> 667
<212> PRT
<213> Nicotiana tabacum

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<400> 6
Met Ala Ala Ala Ala Pro Ser Pro Ser Ser Ser Ala Phe Ser Lys Thr
1          5          10          15
Leu Ser Pro Ser Ser Ser Thr Ser Ser Thr Leu Leu Pro Arg Ser Thr
          20          25          30
Phe Pro Phe Pro His His Pro His Lys Thr Thr Pro Pro Pro Leu His
          35          40          45
Leu Thr His Thr His Ile His Ile His Ser Gln Arg Arg Arg Phe Thr
          50          55          60
Ile Ser Asn Val Ile Ser Thr Asn Gln Lys Val Ser Gln Thr Glu Lys
65          70          75          80
Thr Glu Thr Phe Val Ser Arg Phe Ala Pro Asp Glu Pro Arg Lys Gly
          85          90          95
Ser Asp Val Leu Val Glu Ala Leu Glu Arg Glu Gly Val Thr Asp Val
          100          105          110
Phe Ala Tyr Pro Gly Gly Ala Ser Met Glu Ile His Gln Ala Leu Thr
          115          120          125
Arg Ser Ser Ile Ile Arg Asn Val Leu Pro Arg His Glu Gln Gly Gly
          130          135          140
Val Phe Ala Ala Glu Gly Tyr Ala Arg Ala Thr Gly Phe Pro Gly Val
          145          150          155          160
Cys Ile Ala Thr Ser Gly Pro Gly Ala Thr Asn Leu Val Ser Gly Leu
          165          170          175
Ala Asp Ala Leu Leu Asp Ser Val Pro Ile Val Ala Ile Thr Gly Gln
          180          185          190
Val Pro Arg Arg Met Ile Gly Thr Asp Ala Phe Gln Glu Thr Pro Ile
          195          200          205
Val Glu Val Thr Arg Ser Ile Thr Lys His Asn Tyr Leu Val Met Asp
          210          215          220
Val Glu Asp Ile Pro Arg Val Val Arg Glu Ala Phe Phe Leu Ala Arg
          225          230          235          240
Ser Gly Arg Pro Gly Pro Ile Leu Ile Asp Val Pro Lys Asp Ile Gln
          245          250          255
Gln Gln Leu Val Ile Pro Asp Trp Asp Gln Pro Met Arg Leu Pro Gly
          260          265          270
Tyr Met Ser Arg Leu Pro Lys Leu Pro Asn Glu Met Leu Leu Glu Gln
          275          280          285

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49j

Ile	Val	Arg	Leu	Ile	Ser	Glu	Ser	Lys	Lys	Pro	Val	Leu	Tyr	Val	Gly
	290					295				300					
Gly	Gly	Cys	Ser	Gln	Ser	Ser	Glu	Asp	Leu	Arg	Arg	Phe	Val	Glu	Leu
305				310					315						320
Thr	Gly	Ile	Pro	Val	Ala	Ser	Thr	Leu	Met	Gly	Leu	Gly	Ala	Phe	Pro
			325					330						335	
Thr	Gly	Asp	Glu	Leu	Ser	Leu	Ser	Met	Leu	Gly	Met	His	Gly	Thr	Val
		340					345					350			
Tyr	Ala	Asn	Tyr	Ala	Val	Asp	Ser	Ser	Asp	Leu	Leu	Leu	Ala	Phe	Gly
	355					360				365					
Val	Arg	Phe	Asp	Asp	Arg	Val	Thr	Gly	Lys	Leu	Glu	Ala	Phe	Ala	Ser
	370					375				380					
Arg	Ala	Lys	Ile	Val	His	Ile	Asp	Ile	Asp	Ser	Ala	Glu	Ile	Gly	Lys
385				390					395						400
Asn	Lys	Gln	Pro	His	Val	Ser	Ile	Cys	Ala	Asp	Ile	Lys	Leu	Ala	Leu
			405					410					415		
Gln	Gly	Leu	Asn	Ser	Ile	Leu	Glu	Ser	Lys	Glu	Gly	Lys	Leu	Lys	Leu
		420					425					430			
Asp	Phe	Ser	Ala	Trp	Arg	Gln	Glu	Leu	Thr	Glu	Gln	Lys	Val	Lys	His
	435					440				445					
Pro	Leu	Asn	Phe	Lys	Thr	Phe	Gly	Asp	Ala	Ile	Pro	Pro	Gln	Tyr	Ala
	450					455				460					
Ile	Gln	Val	Leu	Asp	Glu	Leu	Thr	Asn	Gly	Asn	Ala	Ile	Ile	Ser	Thr
465				470					475						480
Gly	Val	Gly	Gln	His	Gln	Met	Trp	Ala	Ala	Gln	Tyr	Tyr	Lys	Tyr	Arg
			485					490					495		
Lys	Pro	Arg	Gln	Trp	Leu	Thr	Ser	Gly	Gly	Leu	Gly	Ala	Met	Gly	Phe
		500						505				510			
Gly	Leu	Pro	Ala	Ala	Ile	Gly	Ala	Ala	Val	Gly	Arg	Pro	Asp	Glu	Val
	515					520				525					
Val	Val	Asp	Ile	Asp	Gly	Asp	Gly	Ser	Phe	Ile	Met	Asn	Val	Gln	Glu
	530				535					540					
Leu	Ala	Thr	Ile	Lys	Val	Glu	Asn	Leu	Pro	Val	Lys	Ile	Met	Leu	Leu
545				550					555						560
Asn	Asn	Gln	His	Leu	Gly	Met	Val	Val	Gln	Trp	Glu	Asp	Arg	Phe	Tyr
			565					570					575		
Lys	Ala	Asn	Arg	Ala	His	Thr	Tyr	Leu	Gly	Asn	Pro	Ser	Asn	Glu	Ala
		580					585					590			
Glu	Ile	Phe	Pro	Asn	Met	Leu	Lys	Phe	Ala	Glu	Ala	Cys	Gly	Val	Pro
	595					600						605			
Ala	Ala	Arg	Val	Thr	His	Arg	Asp	Asp	Leu	Arg	Ala	Ala	Ile	Gln	Lys
	610					615					620				
Met	Leu	Asp	Thr	Pro	Gly	Pro	Tyr	Leu	Leu	Asp	Val	Ile	Val	Pro	His
625				630						635					640
Gln	Glu	His	Val	Leu	Pro	Met	Ile	Pro	Ser	Gly	Gly	Ala	Phe	Lys	Asp
			645					650					655		
Val	Ile	Thr	Glu	Gly	Asp	Gly	Arg	Ser	Ser	Tyr					
		660						665							

<210> 7

<211> 664

<212> PRT

<213> Nicotiana tabacum

49k

<400> 7

Met	Ala	Ala	Ala	Ala	Ala	Ala	Pro	Ser	Pro	Ser	Phe	Ser	Lys	Thr	Leu	1	5	10	15
Ser	Ser	Ser	Ser	Ser	Lys	Ser	Ser	Thr	Leu	Leu	Pro	Arg	Ser	Thr	Phe	20	25	30	
Pro	Phe	Pro	His	His	Pro	His	Lys	Thr	Thr	Pro	Pro	Pro	Leu	His	Leu	35	40	45	
Thr	Pro	Thr	His	Ile	His	Ser	Gln	Arg	Arg	Arg	Phe	Thr	Ile	Ser	Asn	50	55	60	
Val	Ile	Ser	Thr	Thr	Gln	Lys	Val	Ser	Glu	Thr	Gln	Lys	Ala	Glu	Thr	65	70	75	80
Phe	Val	Ser	Arg	Phe	Ala	Pro	Asp	Glu	Pro	Arg	Lys	Gly	Ser	Asp	Val	85	90	95	
Leu	Val	Glu	Ala	Leu	Glu	Arg	Glu	Gly	Val	Thr	Asp	Val	Phe	Ala	Tyr	100	105	110	
Pro	Gly	Gly	Ala	Ser	Met	Glu	Ile	His	Gln	Ala	Leu	Thr	Arg	Ser	Ser	115	120	125	
Ile	Ile	Arg	Asn	Val	Leu	Pro	Arg	His	Glu	Gln	Gly	Gly	Val	Phe	Ala	130	135	140	
Ala	Glu	Gly	Tyr	Ala	Arg	Ala	Thr	Gly	Phe	Pro	Gly	Val	Cys	Ile	Ala	145	150	155	160
Thr	Ser	Gly	Pro	Gly	Ala	Thr	Asn	Leu	Val	Ser	Gly	Leu	Ala	Asp	Ala	165	170	175	
Leu	Leu	Asp	Ser	Val	Pro	Ile	Val	Ala	Ile	Thr	Gly	Gln	Val	Pro	Arg	180	185	190	
Arg	Met	Ile	Gly	Thr	Asp	Ala	Phe	Gln	Glu	Thr	Pro	Ile	Val	Glu	Val	195	200	205	
Thr	Arg	Ser	Ile	Thr	Lys	His	Asn	Tyr	Leu	Val	Met	Asp	Val	Glu	Asp	210	215	220	
Ile	Pro	Arg	Val	Val	Arg	Glu	Ala	Phe	Phe	Leu	Ala	Arg	Ser	Gly	Arg	225	230	235	240
Pro	Gly	Pro	Val	Leu	Ile	Asp	Val	Pro	Lys	Asp	Ile	Gln	Gln	Gln	Leu	245	250	255	
Val	Ile	Pro	Asp	Trp	Asp	Gln	Pro	Met	Arg	Leu	Pro	Gly	Tyr	Met	Ser	260	265	270	
Arg	Leu	Pro	Lys	Leu	Pro	Asn	Glu	Met	Leu	Leu	Glu	Gln	Ile	Val	Arg	275	280	285	
Leu	Ile	Ser	Glu	Ser	Lys	Lys	Pro	Val	Leu	Tyr	Val	Gly	Gly	Gly	Cys	290	295	300	
Ser	Gln	Ser	Ser	Glu	Glu	Leu	Arg	Arg	Phe	Val	Glu	Leu	Thr	Gly	Ile	305	310	315	320
Pro	Val	Ala	Ser	Thr	Leu	Met	Gly	Leu	Gly	Ala	Phe	Pro	Thr	Gly	Asp	325	330	335	
Glu	Leu	Ser	Leu	Ser	Met	Leu	Gly	Met	His	Gly	Thr	Val	Tyr	Ala	Asn	340	345	350	
Tyr	Ala	Val	Asp	Ser	Ser	Asp	Leu	Leu	Leu	Ala	Phe	Gly	Val	Arg	Phe	355	360	365	
Asp	Asp	Arg	Val	Thr	Gly	Lys	Leu	Glu	Ala	Phe	Ala	Ser	Arg	Ala	Lys	370	375	380	
Ile	Val	His	Ile	Asp	Ile	Asp	Ser	Ala	Glu	Ile	Gly	Lys	Asn	Lys	Gln	385	390	395	400
Pro	His	Val	Ser	Ile	Cys	Ala	Asp	Ile	Lys	Leu	Ala	Leu	Gln	Gly	Leu	405	410	415	
Asn	Ser	Ile	Leu	Glu	Ser	Lys	Glu	Gly	Lys	Leu	Lys	Leu	Asp	Phe	Ser	420	425	430	

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<210> 8
<211> 671
<212> PRT
<213> Arabidopsis thaliana
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<400>	8															
Met	Ala	Ala	Ala	Thr	Thr	Thr	Thr	Thr	Thr	Ser	Ser	Ser	Ile	Ser	Phe	
1				5					10					15		
Ser	Thr	Lys	Pro	Ser	Pro	Ser	Ser	Ser	Lys	Ser	Pro	Leu	Pro	Ile	Ser	
			20					25					30			
Arg	Phe	Ser	Leu	Pro	Phe	Ser	Leu	Asn	Pro	Asn	Lys	Ser	Ser	Ser	Ser	
		35					40					45				
Ser	Arg	Arg	Arg	Gly	Ile	Lys	Ser	Ser	Ser	Pro	Ser	Ser	Ile	Ser	Ala	
	50					55					60					
Val	Leu	Asn	Thr	Thr	Thr	Asn	Val	Thr	Thr	Thr	Pro	Ser	Pro	Thr	Lys	
65					70					75					80	
Pro	Thr	Lys	Pro	Glu	Thr	Phe	Ile	Ser	Arg	Phe	Ala	Pro	Asp	Gln	Pro	
				85					90					95		
Arg	Lys	Gly	Ala	Asp	Ile	Leu	Val	Glu	Ala	Leu	Glu	Arg	Gln	Gly	Val	
			100					105					110			
Glu	Thr	Val	Phe	Ala	Tyr	Pro	Gly	Gly	Ala	Ser	Met	Glu	Ile	His	Gln	
		115					120					125				
Ala	Leu	Thr	Arg	Ser	Ser	Ser	Ile	Arg	Asn	Val	Leu	Pro	Arg	His	Glu	
	130					135					140					

49m

Gln	Gly	Gly	Val	Phe	Ala	Ala	Glu	Gly	Tyr	Ala	Arg	Ser	Ser	Gly	Lys	145	150	155	160
Pro	Gly	Ile	Cys	Ile	Ala	Thr	Ser	Gly	Pro	Gly	Ala	Thr	Asn	Leu	Val	165	170	175	
Ser	Gly	Leu	Ala	Asp	Ala	Leu	Leu	Asp	Ser	Val	Pro	Leu	Val	Ala	Ile	180	185	190	
Thr	Gly	Gln	Val	Pro	Arg	Arg	Met	Ile	Gly	Thr	Asp	Ala	Phe	Gln	Glu	195	200	205	
Thr	Pro	Ile	Val	Glu	Val	Thr	Arg	Ser	Ile	Thr	Lys	His	Asn	Tyr	Leu	210	215	220	
Val	Met	Asp	Val	Glu	Asp	Ile	Pro	Arg	Ile	Ile	Glu	Glu	Ala	Phe	Phe	225	230	235	240
Leu	Ala	Thr	Ser	Gly	Arg	Pro	Gly	Pro	Val	Leu	Val	Asp	Val	Pro	Lys	245	250	255	
Asp	Ile	Gln	Gln	Gln	Leu	Ala	Ile	Pro	Asn	Trp	Glu	Gln	Ala	Met	Arg	260	265	270	
Leu	Pro	Gly	Tyr	Met	Ser	Arg	Met	Pro	Lys	Pro	Pro	Glu	Asp	Ser	His	275	280	285	
Leu	Glu	Gln	Ile	Val	Arg	Leu	Ile	Ser	Glu	Ser	Lys	Lys	Pro	Val	Leu	290	295	300	
Tyr	Val	Gly	Gly	Gly	Cys	Leu	Asn	Ser	Ser	Asp	Glu	Leu	Gly	Arg	Phe	305	310	315	320
Val	Glu	Leu	Thr	Gly	Ile	Pro	Val	Ala	Ser	Thr	Leu	Met	Gly	Leu	Gly	325	330	335	
Ser	Tyr	Pro	Cys	Asp	Asp	Glu	Leu	Ser	Leu	His	Met	Leu	Gly	Met	His	340	345	350	
Gly	Thr	Val	Tyr	Ala	Asn	Tyr	Ala	Val	Glu	His	Ser	Asp	Leu	Leu	Leu	355	360	365	
Ala	Phe	Gly	Val	Arg	Phe	Asp	Asp	Arg	Val	Thr	Gly	Lys	Leu	Glu	Ala	370	375	380	
Phe	Ala	Ser	Arg	Ala	Lys	Ile	Val	His	Ile	Asp	Ile	Asp	Ser	Ala	Glu	385	390	395	400
Ile	Gly	Lys	Asn	Lys	Thr	Pro	His	Val	Ser	Val	Cys	Gly	Asp	Val	Lys	405	410	415	
Leu	Ala	Leu	Gln	Gly	Met	Asn	Lys	Val	Leu	Glu	Asn	Arg	Ala	Glu	Glu	420	425	430	
Leu	Lys	Leu	Asp	Phe	Gly	Val	Trp	Arg	Asn	Glu	Leu	Asn	Val	Gln	Lys	435	440	445	
Gln	Lys	Phe	Pro	Leu	Ser	Phe	Lys	Thr	Phe	Gly	Glu	Ala	Ile	Pro	Pro	450	455	460	
Gln	Tyr	Ala	Ile	Lys	Val	Leu	Asp	Glu	Leu	Thr	Asp	Gly	Lys	Ala	Ile	465	470	475	480
Ile	Ser	Thr	Gly	Val	Gly	Gln	His	Gln	Met	Trp	Ala	Ala	Gln	Phe	Tyr	485	490	495	
Asn	Tyr	Lys	Lys	Pro	Arg	Arg	Gln	Trp	Leu	Ser	Ser	Gly	Gly	Leu	Gly	500	505	510	
Ala	Met	Gly	Phe	Gly	Leu	Pro	Ala	Ala	Ile	Gly	Ala	Ser	Val	Ala	Asn	515	520	525	
Pro	Asp	Ala	Ile	Val	Val	Asp	Ile	Asp	Gly	Asp	Gly	Ser	Phe	Ile	Met	530	535	540	
Asn	Val	Gln	Glu	Leu	Ala	Thr	Ile	Arg	Val	Glu	Asn	Leu	Pro	Val	Lys	545	550	555	560
Val	Leu	Leu	Leu	Asn	Asn	Gln	His	Leu	Gly	Met	Val	Met	Gln	Trp	Glu	565	570	575	
Asp	Arg	Phe	Tyr	Lys	Ala	Asn	Arg	Ala	His	Thr	Phe	Leu	Gly	Asp	Pro	580	585	590	

49n

Ala	Gln	Glu	Asp	Glu	Ile	Phe	Pro	Asn	Met	Leu	Leu	Phe	Ala	Ala	Ala
		595					600					605			
Cys	Gly	Ile	Pro	Ala	Ala	Arg	Val	Thr	Lys	Lys	Ala	Asp	Leu	Arg	Glu
	610					615					620				
Ala	Ile	Gln	Thr	Met	Leu	Asp	Thr	Pro	Gly	Pro	Tyr	Leu	Leu	Asp	Val
625					630					635					640
Ile	Cys	Pro	His	Gln	Glu	His	Val	Leu	Pro	Met	Ile	Pro	Asn	Gly	Gly
				645					650					655	
Thr	Phe	Asn	Asp	Val	Ile	Thr	Glu	Gly	Asp	Gly	Arg	Ile	Lys	Tyr	
			660					665					670		

<210> 9
 <211> 652
 <212> PRT
 <213> Brassica napus

<400> 9

Met	Ala	Ala	Ala	Thr	Ser	Ser	Ser	Pro	Ile	Ser	Leu	Thr	Ala	Lys	Pro
1				5					10					15	
Ser	Ser	Lys	Ser	Pro	Leu	Pro	Ile	Ser	Arg	Phe	Ser	Leu	Pro	Phe	Ser
		20						25					30		
Leu	Thr	Pro	Gln	Lys	Pro	Ser	Ser	Arg	Leu	His	Arg	Pro	Leu	Ala	Ile
		35					40					45			
Ser	Ala	Val	Leu	Asn	Ser	Pro	Val	Asn	Val	Ala	Pro	Glu	Lys	Thr	Asp
	50				55					60					
Lys	Ile	Lys	Thr	Phe	Ile	Ser	Arg	Tyr	Ala	Pro	Asp	Glu	Pro	Arg	Lys
65					70				75						80
Gly	Ala	Asp	Ile	Leu	Val	Glu	Ala	Leu	Glu	Arg	Gln	Gly	Val	Glu	Thr
				85				90						95	
Val	Phe	Ala	Tyr	Pro	Gly	Gly	Ala	Ser	Met	Glu	Ile	His	Gln	Ala	Leu
			100					105					110		
Thr	Arg	Ser	Ser	Thr	Ile	Arg	Asn	Val	Leu	Pro	Arg	His	Glu	Gln	Gly
		115				120						125			
Gly	Val	Phe	Ala	Ala	Glu	Gly	Tyr	Ala	Arg	Ser	Ser	Gly	Lys	Pro	Gly
	130					135					140				
Ile	Cys	Ile	Ala	Thr	Ser	Gly	Pro	Gly	Ala	Thr	Asn	Leu	Val	Ser	Gly
145					150					155					160
Leu	Ala	Asp	Ala	Met	Leu	Asp	Ser	Val	Pro	Leu	Val	Ala	Ile	Thr	Gly
				165				170						175	
Gln	Val	Pro	Arg	Arg	Met	Ile	Gly	Thr	Asp	Ala	Phe	Gln	Glu	Thr	Pro
			180					185					190		
Ile	Val	Glu	Val	Thr	Arg	Ser	Ile	Thr	Lys	His	Asn	Tyr	Leu	Val	Met
	195					200						205			
Asp	Val	Asp	Asp	Ile	Pro	Arg	Ile	Val	Gln	Glu	Ala	Phe	Phe	Leu	Ala
	210				215						220				
Thr	Ser	Gly	Arg	Pro	Gly	Pro	Val	Leu	Val	Asp	Val	Pro	Lys	Asp	Ile
225					230					235					240
Gln	Gln	Gln	Leu	Ala	Ile	Pro	Asn	Trp	Asp	Gln	Pro	Met	Arg	Leu	Pro
				245				250						255	
Gly	Tyr	Met	Ser	Arg	Leu	Pro	Gln	Pro	Pro	Glu	Val	Ser	Gln	Leu	Gly
		260						265					270		
Gln	Ile	Val	Arg	Leu	Ile	Ser	Glu	Ser	Lys	Arg	Pro	Val	Leu	Tyr	Val
	275					280						285			
Gly	Gly	Gly	Ser	Leu	Asn	Ser	Ser	Glu	Glu	Leu	Gly	Arg	Phe	Val	Glu
	290					295					300				

490

Leu Thr Gly Ile Pro Val Ala Ser Thr Leu Met Gly Leu Gly Ser Tyr
 305 310 315 320
 Pro Cys Asn Asp Glu Leu Ser Leu Gln Met Leu Gly Met His Gly Thr
 325 330 335
 Val Tyr Ala Asn Tyr Ala Val Glu His Ser Asp Leu Leu Leu Ala Phe
 340 345 350
 Gly Val Arg Phe Asp Asp Arg Val Thr Gly Lys Leu Glu Ala Phe Ala
 355 360 365
 Ser Arg Ala Lys Ile Val His Ile Asp Ile Asp Ser Ala Glu Ile Gly
 370 375 380
 Lys Asn Lys Thr Pro His Val Ser Val Cys Gly Asp Val Lys Leu Ala
 385 390 395 400
 Leu Gln Gly Met Asn Lys Val Leu Glu Asn Arg Ala Glu Glu Leu Lys
 405 410 415
 Leu Asp Phe Gly Val Trp Arg Ser Glu Leu Ser Glu Gln Lys Gln Lys
 420 425 430
 Phe Pro Leu Ser Phe Lys Thr Phe Gly Glu Ala Ile Pro Pro Gln Tyr
 435 440 445
 Ala Ile Gln Val Leu Asp Glu Leu Thr Gln Gly Lys Ala Ile Ile Ser
 450 455 460
 Thr Gly Val Gly Gln His Gln Met Trp Ala Ala Gln Phe Tyr Lys Tyr
 465 470 475 480
 Arg Lys Pro Arg Gln Trp Leu Ser Ser Ser Gly Leu Gly Ala Met Gly
 485 490 495
 Phe Gly Leu Pro Ala Ala Ile Gly Ala Ser Val Ala Asn Pro Asp Ala
 500 505 510
 Ile Val Val Asp Ile Asp Gly Asp Gly Ser Phe Ile Met Asn Val Gln
 515 520 525
 Glu Leu Ala Thr Ile Arg Val Glu Asn Leu Pro Val Lys Ile Leu Leu
 530 535 540
 Leu Asn Asn Gln His Leu Gly Met Val Met Gln Trp Glu Asp Arg Phe
 545 550 555 560
 Tyr Lys Ala Asn Arg Ala His Thr Tyr Leu Gly Asp Pro Ala Arg Glu
 565 570 575
 Asn Glu Ile Phe Pro Asn Met Leu Gln Phe Ala Gly Ala Cys Gly Ile
 580 585 590
 Pro Ala Ala Arg Val Thr Lys Lys Glu Glu Leu Arg Glu Ala Ile Gln
 595 600 605
 Thr Met Leu Asp Thr Pro Gly Pro Tyr Leu Leu Asp Val Ile Cys Pro
 610 615 620
 His Gln Glu His Val Leu Pro Met Ile Pro Ser Gly Gly Thr Phe Lys
 625 630 635 640
 Asp Val Ile Thr Glu Gly Asp Gly Arg Thr Lys Tyr
 645 650

<210> 10
 <211> 637
 <212> PRT
 <213> Brassica napus

<400> 10
 Met Ala Ser Phe Ser Phe Phe Gly Thr Ile Pro Ser Ser Pro Thr Lys
 1 5 10 15
 Ala Ser Val Phe Ser Leu Pro Val Ser Val Thr Thr Leu Pro Ser Phe
 20 25 30

49p

Pro	Arg	Arg	Arg	Ala	Thr	Arg	Val	Ser	Val	Ser	Ala	Asn	Ser	Lys	Lys
		35					40					45			
Asp	Gln	Asp	Arg	Thr	Ala	Ser	Arg	Arg	Glu	Asn	Pro	Ser	Thr	Phe	Ser
	50					55				60					
Ser	Lys	Tyr	Ala	Pro	Asn	Val	Pro	Arg	Ser	Gly	Ala	Asp	Ile	Leu	Val
65					70					75				80	
Glu	Ala	Leu	Glu	Arg	Gln	Gly	Val	Asp	Val	Val	Phe	Ala	Tyr	Pro	Gly
				85					90					95	
Gly	Ala	Ser	Met	Glu	Ile	His	Gln	Ala	Leu	Thr	Arg	Ser	Asn	Thr	Ile
			100					105					110		
Arg	Asn	Val	Leu	Pro	Arg	His	Glu	Gln	Gly	Gly	Ile	Phe	Ala	Ala	Glu
		115					120					125			
Gly	Tyr	Ala	Arg	Ser	Ser	Gly	Lys	Pro	Gly	Ile	Cys	Ile	Ala	Thr	Ser
	130					135					140				
Gly	Pro	Gly	Ala	Met	Asn	Leu	Val	Ser	Gly	Leu	Ala	Asp	Ala	Leu	Phe
145					150					155				160	
Asp	Ser	Val	Pro	Leu	Ile	Ala	Ile	Thr	Gly	Gln	Val	Pro	Arg	Arg	Met
				165					170					175	
Ile	Gly	Thr	Met	Ala	Phe	Gln	Glu	Thr	Pro	Val	Val	Glu	Val	Thr	Arg
			180					185						190	
Thr	Ile	Thr	Lys	His	Asn	Tyr	Leu	Val	Met	Glu	Val	Asp	Asp	Ile	Pro
		195					200					205			
Arg	Ile	Val	Arg	Glu	Ala	Phe	Phe	Leu	Ala	Thr	Ser	Val	Arg	Pro	Gly
	210					215						220			
Pro	Val	Leu	Ile	Asp	Val	Pro	Lys	Asp	Val	Gln	Gln	Gln	Phe	Ala	Ile
225					230					235				240	
Pro	Asn	Trp	Glu	Gln	Pro	Met	Arg	Leu	Pro	Leu	Tyr	Met	Ser	Thr	Met
				245					250					255	
Pro	Lys	Pro	Pro	Lys	Val	Ser	His	Leu	Glu	Gln	Ile	Leu	Arg	Leu	Val
			260					265					270		
Ser	Glu	Ser	Lys	Arg	Pro	Val	Leu	Tyr	Val	Gly	Gly	Gly	Cys	Leu	Asn
		275					280					285			
Ser	Ser	Glu	Glu	Leu	Arg	Arg	Phe	Val	Glu	Leu	Thr	Gly	Ile	Pro	Val
	290					295					300				
Ala	Ser	Thr	Phe	Met	Gly	Leu	Gly	Ser	Tyr	Pro	Cys	Asp	Asp	Glu	Glu
305					310					315				320	
Phe	Ser	Leu	Gln	Met	Leu	Gly	Met	His	Gly	Thr	Val	Tyr	Ala	Asn	Tyr
				325					330					335	
Ala	Val	Glu	Tyr	Ser	Asp	Leu	Leu	Leu	Ala	Phe	Gly	Val	Arg	Phe	Asp
			340					345					350		
Asp	Arg	Val	Thr	Gly	Lys	Leu	Glu	Ala	Phe	Ala	Ser	Arg	Ala	Lys	Ile
		355					360					365			
Val	His	Ile	Asp	Ile	Asp	Ser	Thr	Glu	Ile	Gly	Lys	Asn	Lys	Thr	Pro
	370					375					380				
His	Val	Ser	Val	Cys	Cys	Asp	Val	Gln	Leu	Ala	Leu	Gln	Gly	Met	Asn
385					390					395				400	
Glu	Val	Leu	Glu	Asn	Arg	Arg	Asp	Val	Leu	Asp	Phe	Gly	Glu	Trp	Arg
				405					410					415	
Cys	Glu	Leu	Asn	Glu	Gln	Arg	Leu	Lys	Phe	Pro	Leu	Arg	Tyr	Lys	Thr
			420					425					430		
Phe	Gly	Glu	Glu	Ile	Pro	Pro	Gln	Tyr	Ala	Ile	Gln	Leu	Leu	Asp	Glu
		435					440					445			
Leu	Thr	Asp	Gly	Lys	Ala	Ile	Ile	Thr	Thr	Gly	Val	Gly	Gln	His	Gln
	450					455					460				
Met	Trp	Ala	Ala	Gln	Phe	Tyr	Arg	Phe	Lys	Lys	Pro	Arg	Gln	Trp	Leu
465					470					475				480	

49q

Ser	Ser	Gly	Gly	Leu	Gly	Ala	Met	Gly	Phe	Gly	Leu	Pro	Ala	Ala	Met			
				485					490						495			
Gly	Ala	Ala	Ile	Ala	Asn	Pro	Gly	Ala	Val	Val	Val	Asp	Ile	Asp	Gly			
			500					505						510				
Asp	Gly	Ser	Phe	Ile	Met	Asn	Ile	Gln	Glu	Leu	Ala	Thr	Ile	Arg	Val			
		515					520					525						
Glu	Asn	Leu	Pro	Val	Lys	Val	Leu	Leu	Ile	Asn	Asn	Gln	His	Leu	Gly			
	530					535					540							
Met	Val	Leu	Gln	Trp	Glu	Asp	His	Phe	Tyr	Ala	Ala	Asn	Arg	Ala	Asp			
545					550					555					560			
Ser	Phe	Leu	Gly	Asp	Pro	Ala	Asn	Pro	Glu	Ala	Val	Phe	Pro	Asp	Met			
			565						570					575				
Leu	Leu	Phe	Ala	Ala	Ser	Cys	Gly	Ile	Pro	Ala	Ala	Arg	Val	Thr	Arg			
		580					585						590					
Arg	Glu	Asp	Leu	Arg	Glu	Ala	Ile	Gln	Thr	Met	Leu	Asp	Thr	Pro	Gly			
	595					600						605						
Pro	Phe	Leu	Leu	Asp	Val	Val	Cys	Pro	His	Gln	Asp	His	Val	Leu	Pro			
	610					615					620							
Leu	Ile	Pro	Ser	Gly	Gly	Thr	Phe	Lys	Asp	Ile	Ile	Val						
625				630						635								

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CLAIMS:

1. A method for the production of an imidazolinone and/or sulfonylurea based resistant acetohydroxy acid synthase (AHAS) variant protein using structure-based
5 information, said method comprising:

(a) aligning a target AHAS protein on pyruvate oxidase template to derive the three-dimensional structure of said target AHAS protein;

(b) modelling one or more imidazolinone and/or
10 sulfonylurea based herbicides into said three-dimensional structure to localize an imidazolinone and/or sulfonylurea based herbicide binding pocket in said target AHAS protein;

(c) selecting as a target for a mutation an amino acid position in said target AHAS protein, wherein said
15 mutation alters the affinity of at least one imidazolinone and/or sulfonylurea based herbicide for said binding pocket;

(d) mutating DNA encoding said target AHAS protein to produce a mutated DNA encoding a variant AHAS containing said mutation at said position; and

(e) expressing said mutated DNA in a first cell,
20 under conditions in which said variant AHAS containing said mutation at said position is produced.

2. The method according to claim 1, further comprising:

(f) expressing DNA encoding wild-type AHAS in
25 parallel in a second cell;

(g) purifying said wild-type and said variant AHAS proteins from said cells;

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(h) assaying said wild-type and said variant AHAS proteins for catalytic activity in the conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the
5 absence and in the presence of said imidazolinone or sulfonylurea based herbicide; and

(i) repeating steps (c)-(h), wherein said DNA encoding said variant of step (e) is used as the AHAS-encoding DNA in step (c) until an imidazolinone or sulfonylurea based
10 herbicide resistant AHAS variant protein is identified having:

(i) in the absence of said imidazolinone or sulfonylurea based herbicide,

(a) a catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

15 (b) catalytic activity in combination with any imidazolinone or sulfonylurea based herbicide resistant AHAS variant protein also expressed in said cell, which may be the same as or different than said first AHAS variant protein, sufficient to maintain the viability of a cell in
20 which it is expressed; wherein said cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to an imidazolinone or sulfonylurea based herbicide than is wild-type AHAS.

25 3. The method according to claim 2, wherein said catalytic activity in the absence of an imidazolinone or sulfonylurea based herbicide is more than 20% of the catalytic activity of said wild-type AHAS in the absence of an imidazolinone or sulfonylurea based herbicide.

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4. The method according to claim 3, wherein the herbicide is an imidazolinone herbicide and said herbicide-resistant AHAS variant protein has:

5 (i) catalytic activity in the absence of said herbicide of more than 20% of the catalytic activity of said wild-type AHAS;

(ii) catalytic activity that is more resistant to the presence of imidazolinone herbicides compared to wild-type AHAS; and

10 (iii) catalytic activity that is more sensitive to the presence of sulfonylurea herbicides compared to imidazolinone herbicides.

5. The method according to claim 1, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

15 6. The method according to claim 1, wherein said first cell is *E. coli*.

7. The method according to claim 2, wherein said first and second cells are *E. coli*.

8. The method according to claim 1, wherein said target
20 AHAS protein comprises a protein having the sequence of SEQ ID NO: 1 or the amino acid sequence of another plant AHAS protein.

9. The method according to claim 1, wherein said mutation is a substitution of at least one different amino acid residue at an amino acid residue of the sequence of SEQ ID
25 NO: 1 selected from the group consisting of F135, M53, R128, an amino acid residue of another plant AHAS protein at an amino

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acid aligned with any of the foregoing, and any combination of any of the foregoing substitutions.

10. The method according to claim 9, wherein said substitution is selected from the group consisting of Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, and a combination of any of the foregoing substitutions.

11. A method for the production of an imidazolinone and/or sulfonylurea based resistant acetohydroxy acid synthase (AHAS) variant protein using structure-based information, said method comprising:

(a) aligning a target AHAS protein on a first AHAS template derived from a polypeptide having the sequence of SEQ ID NO: 1 or the amino acid sequence of another plant AHAS protein, to derive the three-dimensional structure of said target AHAS protein;

(b) modelling one or more imidazolinone or sulfonylurea based herbicides into said three-dimensional structure to localize an imidazolinone or sulfonylurea based herbicide binding pocket in said target AHAS protein;

(c) selecting as a target for a mutation, at least one amino acid position in said target AHAS protein, wherein said mutation alters the affinity of at least one imidazolinone or sulfonylurea based herbicide for said binding pocket;

(d) mutating DNA encoding said target AHAS protein to produce a mutated DNA encoding a variant AHAS containing the mutation at said position; and

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(e) expressing said mutated DNA in a first cell, under conditions in which said variant AHAS containing said mutation at said position is produced.

12. The method according to claim 11, further comprising:

5 (f) expressing DNA encoding wild-type AHAS in parallel in a second cell;

(g) purifying said wild-type and said variant AHAS proteins from said cells;

10 (h) assaying said wild-type and said variant AHAS proteins for catalytic activity in conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of said imidazolinone or sulfonylurea based herbicide; and

15 (i) repeating steps (c)-(h), wherein said DNA encoding the variant of step (e) is used as the AHAS-encoding DNA in step (c) until a first herbicide resistant AHAS variant protein is identified having:

20 (i) in the absence of said imidazolinone or sulfonylurea based herbicide,

(a) a catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

25 (b) catalytic activity in combination with any imidazolinone or sulfonylurea based herbicide resistant AHAS variant protein also expressed in said cell, which may be the same as or different than said first AHAS variant protein, sufficient to maintain the viability of a cell in which it is

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expressed; wherein said cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to at least one imidazolinone or sulfonylurea based herbicide than is
5 wild type AHAS.

13. A method for the production of imidazolinone and/or sulfonylurea based herbicide resistant acetohydroxy acid synthase (AHAS) variant protein using structure-based information, said method comprising:

10 (a) aligning a target AHAS protein on a first AHAS template having an identified imidazolinone and/or sulfonylurea based herbicide binding pocket and having the sequence of SEQ ID NO: 1 to derive the three-dimensional structure of said target AHAS protein;

15 (b) selecting as a target for a mutation, at least one amino acid position in said target AHAS protein, wherein said mutation alters the affinity of an imidazolinone or sulfonylurea based herbicide for said binding pocket;

(c) mutating DNA encoding said target AHAS protein to
20 produce a mutated DNA encoding a variant AHAS containing said mutation at said position; and

(d) expressing said mutated DNA in a first cell, under conditions in which said variant AHAS containing said mutation at said position is produced.

25 14. The method according to claim 13, further comprising:

(e) expressing DNA encoding wild-type AHAS in parallel in a second cell;

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(f) purifying said wild-type and said variant AHAS proteins from said cells;

(g) assaying said wild-type and said variant AHAS proteins for catalytic activity in conversion of pyruvate to
5 acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of said imidazolinone or sulfonylurea based herbicide; and

(h) repeating steps (b)-(g), wherein said DNA
10 encoding said AHAS of step (d) is used as the AHAS-encoding DNA in step (b) until a first herbicide resistant AHAS variant protein is identified having:

(i) in the absence of said imidazolinone or sulfonylurea based herbicide,

15 (a) a catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(b) catalytic activity in combination with any imidazolinone or sulfonylurea based herbicide resistant AHAS variant protein also expressed in said cell, which may be the
20 same as or different than said first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed; wherein said cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to at
25 least one imidazolinone or sulfonylurea based herbicide than is wild-type AHAS.

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15. The method according to claim 12 or 14, wherein said catalytic activity in the absence of said at least one imidazolinone or sulfonylurea based herbicide is more than 20% of the catalytic activity of said wild-type AHAS.

5 16. The method according to claim 15, wherein said herbicide is an imidazolinone based herbicide and said first herbicide resistant AHAS variant protein has:

(i) catalytic activity in the absence of said herbicide of more than 20% of the catalytic activity of said
10 wild-type AHAS;

(ii) catalytic activity that is more resistant to the presence of imidazolinone herbicides compared to wild-type AHAS; and

(iii) catalytic activity that is more sensitive to
15 the presence of sulfonylurea based herbicides compared to imidazolinone based herbicides.

17. The method according to claim 16, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

18. The method according to claim 11 or 13, wherein said
20 cell is *E. coli*.

19. The method according to claim 12 or 14, wherein said first and second cells are *E. coli*.

20. The method according to claim 11 or 13, wherein said mutation is a substitution of at least one different amino acid
25 residue at an amino acid residue of the sequence of SEQ ID NO: 1 selected from the group consisting of F135, M53, R128, an amino acid residue of another plant AHAS protein at an amino

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acid aligned with any of the foregoing, and any combination of any of the foregoing substitutions.

21. The method according to claim 20, wherein said substitution is selected from the group consisting of Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, an amino acid residue of another plant AHAS protein at an amino acid aligned with any of the foregoing, and a combination of any of the foregoing substitutions.

22. An isolated DNA encoding an acetohydroxy acid synthase (AHAS) variant protein, said variant protein comprising an AHAS protein modified by substitution of at least one different amino acid residue at a position corresponding to an amino acid residue of the sequence of SEQ ID NO: 1 selected from the group consisting of M53, R128, F135, and any combination of any of the foregoing substitutions.

23. The isolated DNA according to claim 22, wherein said modification alters the ability of an imidazolinone and/or sulfonylurea based herbicide to inhibit the enzymatic activity of said protein.

24. The isolated DNA according to claim 23, wherein said AHAS protein is derived from *Arabidopsis thaliana*.

25. The isolated DNA according to claim 24, wherein said substitution is selected from the group consisting of Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg and a combination of any of the foregoing substitutions.

26. The isolated DNA according to claim 25, wherein said variant AHAS protein has

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(a) in the absence of at least one imidazolinone or sulfonylurea based AHAS inhibiting herbicide,

(i) a catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

5 (ii) catalytic activity in combination with any second imidazolinone or sulfonylurea based herbicide resistant AHAS variant protein also expressed in said cell, which may be the same as or different than said AHAS variant protein, sufficient to maintain the viability of a cell in which it is
10 expressed;

 wherein said cell requires AHAS activity for viability; and

(b) catalytic activity that is more resistant to at least one imidazolinone or sulfonylurea based herbicide than is
15 wild-type AHAS.

27. The isolated DNA according to claim 22, wherein said variant AHAS has more than 20% of the catalytic activity of wild-type AHAS.

28. The isolated DNA according to claim 27, wherein said
20 variant AHAS is more resistant to imidazolinone-based herbicides than to sulfonylurea-based herbicides.

29. A DNA vector comprising the DNA sequence of claim 22 operably linked to a transcription regulatory element.

30. A cell comprising an acetohydroxy acid synthase
25 (AHAS) encoding DNA sequence according to claim 22, wherein said cell is selected from the group consisting of bacterial, fungal, plant, insect, and mammalian cells.

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31. The cell according to claim 30, which is a plant cell.

32. Use of the cell according to claim 31 for the production of a seed.

5 33. A variant acetohydroxy acid synthase (AHAS) protein comprising a protein encoded by the isolated DNA according to claim 22.

34. A variant acetohydroxy acid synthase (AHAS) protein comprising an AHAS protein modified by substitution of at least
10 one different amino acid residue at a position corresponding to an amino acid residue of the sequence of SEQ ID NO: 1 selected from the group consisting of M53, R128, F135, an amino acid residue of another plant AHAS protein at an amino acid aligned with any of the foregoing, and any combination of any of the
15 foregoing substitutions.

35. The variant AHAS protein according to claim 34, wherein said modification alters the ability of an imidazolinone or sulfonylurea based herbicide to inhibit the enzymatic activity of said protein.

20 36. The variant AHAS protein according to claim 35, wherein said AHAS protein is derived from *Arabidopsis thaliana*.

37. The variant AHAS protein according to claim 33, wherein said substitution is selected from the group consisting of Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu,
25 Phe135Arg, and a combination of any of the foregoing substitutions.

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38. The variant AHAS protein according to claim 33,
wherein said variant AHAS protein has

(a) in the absence of said at least one AHAS
inhibiting imidazolinone or sulfonylurea based herbicide;

5 (i) a catalytic activity alone sufficient to maintain
the viability of a cell in which it is expressed; or

(ii) catalytic activity in combination with any
second herbicide resistant AHAS variant protein also expressed
in said cell, which may be the same as or different than said
10 AHAS variant protein, sufficient to maintain the viability of a
cell in which it is expressed;

wherein said cell requires AHAS activity for
viability; and

(b) catalytic activity that is more resistant to at
15 least one imidazolinone or sulfonylurea based herbicide than is
wild type AHAS.

39. The variant AHAS protein according to claim 33,
wherein said variant AHAS has more than 20% of the catalytic
activity of wild-type AHAS.

20 40. A method for conferring imidazolinone and/or
sulfonylurea based herbicide resistance on a cell, said method
comprising:

(a) cloning the isolated DNA according to claim 22
into a compatible expression vector; and

25 (b) transforming said DNA into said cell, under
conditions wherein said gene is expressed at sufficient levels

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to confer imidazolinone or sulfonylurea based herbicide resistance on said cell.

41. A cell prepared according to the method of claim 40.

42. Use of the cell according to claim 41 for the
5 production of a plant.

43. The method according to claim 40, wherein said isolated DNA comprises the Arabidopsis thaliana AHAS gene.

44. The method according to claim 40, wherein said cell is a plant cell.

10 45. The method according to claim 44, wherein said cell is in a seed.

46. A method for controlling weeds in a crop, said method comprising cultivating a crop comprising an imidazolinone and/or sulfonylurea based herbicide resistant plant comprising
15 the cell of claim 41, and treating said crop with said imidazolinone and/or sulfonylurea based herbicide.

47. A method for controlling weeds in a crop, said method comprising cultivating a crop comprising an imidazolinone and/or sulfonylurea based herbicide resistant plant comprising
20 the cell of claim 41, and treating said crop with said imidazolinone and/or sulfonylurea based herbicidal composition including said herbicide.

48. A cell transformed with the DNA according to claim 22, wherein said DNA is expressed in said cell such that
25 it confers imidazolinone and/or sulfonylurea based herbicide resistance on said cell.

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49. Use of a cell comprising the DNA according to any one of claims 22-29 for the production of an imidazolinone and/or sulfonylurea based herbicide resistant plant.

50. Use of a plant comprising a cell transformed with the
5 DNA according to any one of claims 22-29 for the production of an imidazolinone and/or sulfonylurea based herbicide resistant plant.

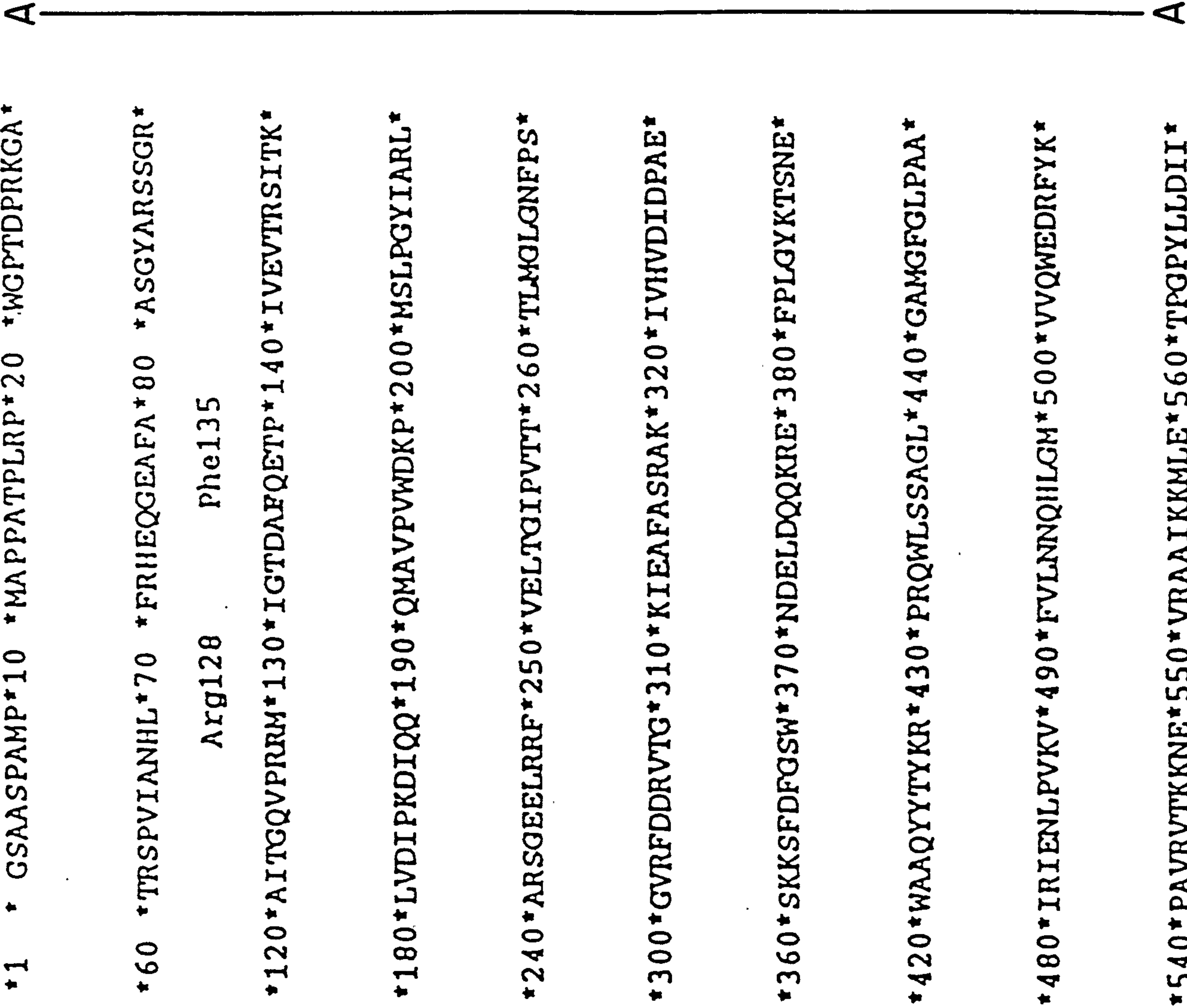


FIG. 1a

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FIG. 1b

Met53

30 *DILVESLERC*40 *GVRDVFAYPG*50 *GASMEIHQAL

90 *VGVCATSGP*100 *GATNLVSALA*110 *DALLDSVPMV

150 *HNYLVLDVDD*160 *IPRVVQEAF*170 *LASSGRPGPV

210 *PKPPATELLE*220 *QVLRVLGESR*230 *RPVLYVGGGC

270 *DDPLSLRMLG*280 *MHGTVYANYA*290 *VDKADLLLAL

330 *IGKNKQPHVS*340 *ICADVKKLALQ*350 *GMNALLEGST

390 *EIQPQYAIQV*400 *LDELTKEAI*410 *IGTGVGQHQM

450 *AGASVANPGV*460 *TVVDIDGDGS*470 *FLMNVQELAM

510 *ANRAHTYLG*520 *PENESEIYPD*530 *FVTIAKGFNI

570 *VPHQEH/LPM*580 *IPSGGAFKDM*590 *ILDGDGRVY

FIG. 2a

SUBSTITUTE SHEET (RULE 26)

POX	* * *	*24 *	TNILAGA*31	*AVIKVLEAWG*41	*VDHLYGIPG*51	*SINSIMDAL			
AHAS_pred	*1	*GSAASPAMP*11	*APPATPLRPW*21	*GPTDPRKGAD*31	*ILVESLERCG*41	*VRDVFAYPG*51	*ASMEIHQALT		
POX	*61	*AERDRIHIYQ*71	*VRHEEVGAMA*81	*AAADAKLTGK*91	*IGVCFGSAGP*101	*GGTHLMNGLY*111	*DAREDHVPVL		
AHAS_pred	*61	*RS PVIANHL*70	*FRHEQGEAFA*80	*ASGYARSSGR*90	*VGVCIAATSGP*100	*GATNLVSALA*110	*DALLDSVPMV		
POX	*121	*ALIGQFGTTG*131	*MNMDTFQEMN*141	*ENPIYADVAD*151	*YNVTAVNAA*161	*LPHVIDEAIR*171	*RAYAHQ GVA		
AHAS_pred	*120	*AITGQVPRRM*130	*IGTDAFQETP*140	*IVEVTRSITK*150	*HNYLVLDVDD*160	*IPRVVQEAF*170	*LASSGRPGPV		
POX	*180	*VVQIPVDLP	*189	*WQQISAEDWY*199	*ASANN	YQT*207	*PLLPEPDVQA*217	*VTRLTQTLLA*227	*AERPLIYYGI
AHAS_pred	*180	*LVDIPKDIQ	*189	*QQMAVPVWD*198	*KPMSLPGYIA*208	*RLPKPPATEL*218	*LEQVLRRLVGE*228	*SRRPVLVYVG	
POX	*237	*GARKAGKELE*247	*QLSKTLKIPL*257	*MSTYPAKGIV*267	*ADRYPAYLGS*277	*ANRVAQKPAN*287	*EALAQADVVL		
AHAS_pred	*238	*GCARSGEELR*248	*RFVELTGIPV*258	*TTTLMGLGNF*268	*PSDDPLSLRM*278	*LGMHGTVYAN*288	*YAVDKADLLL		

A_____A

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FIG. 3a

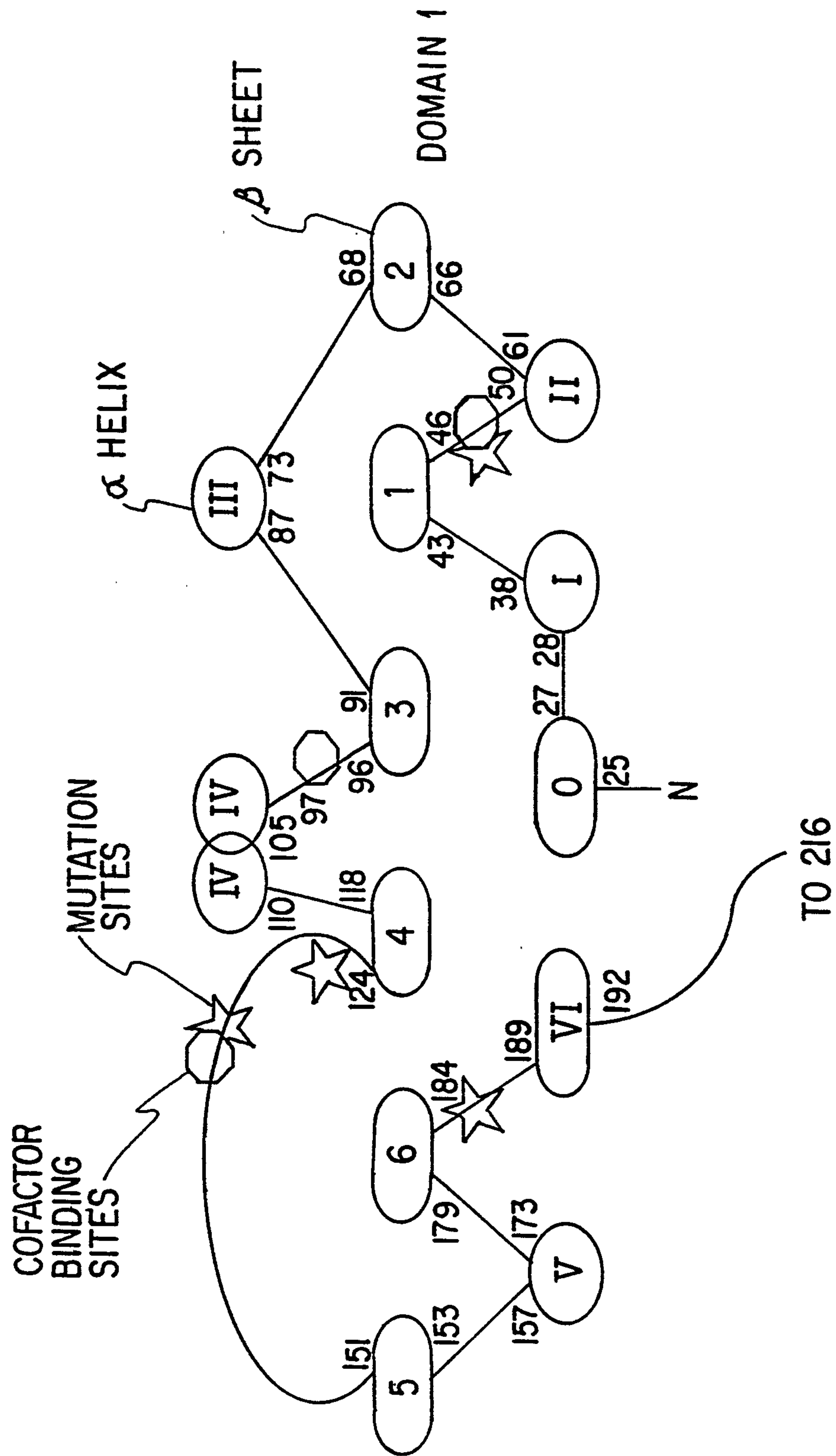


FIG. 3b

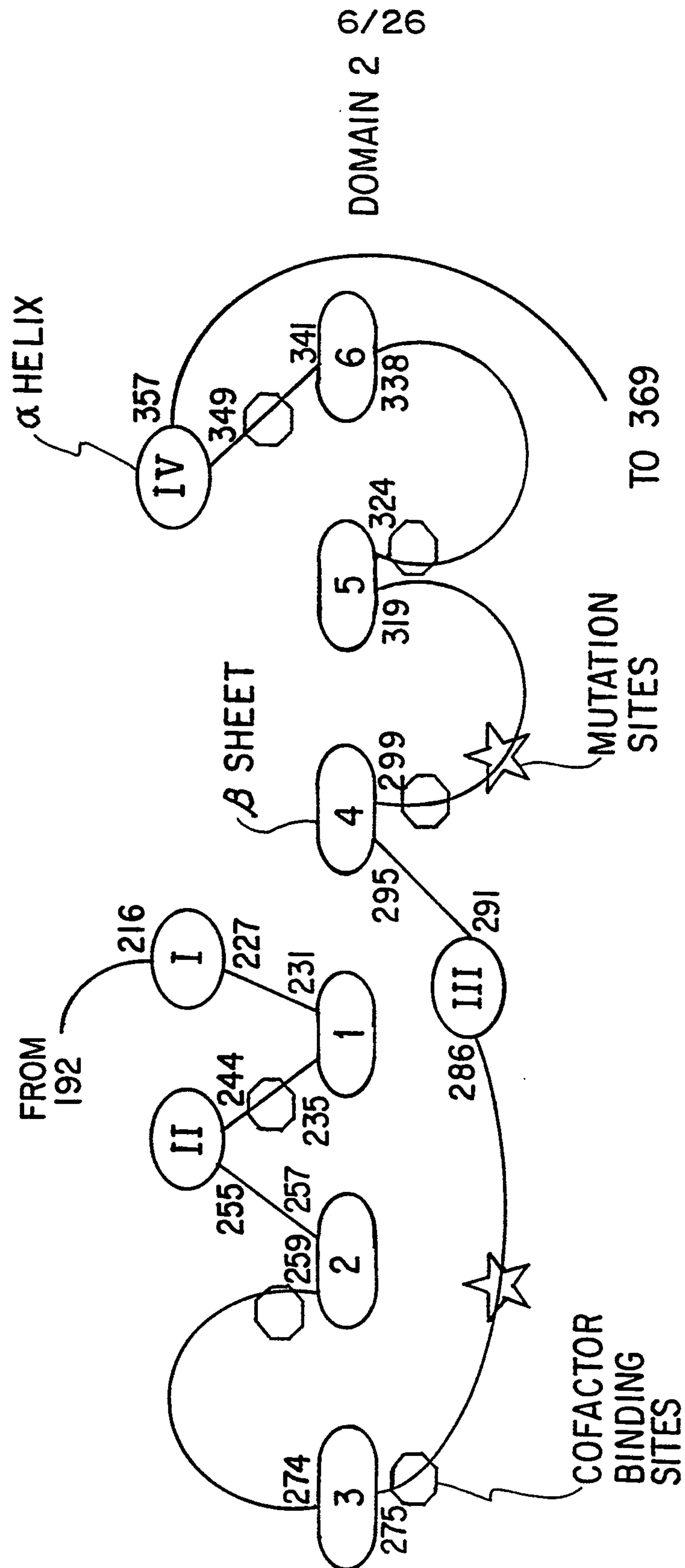
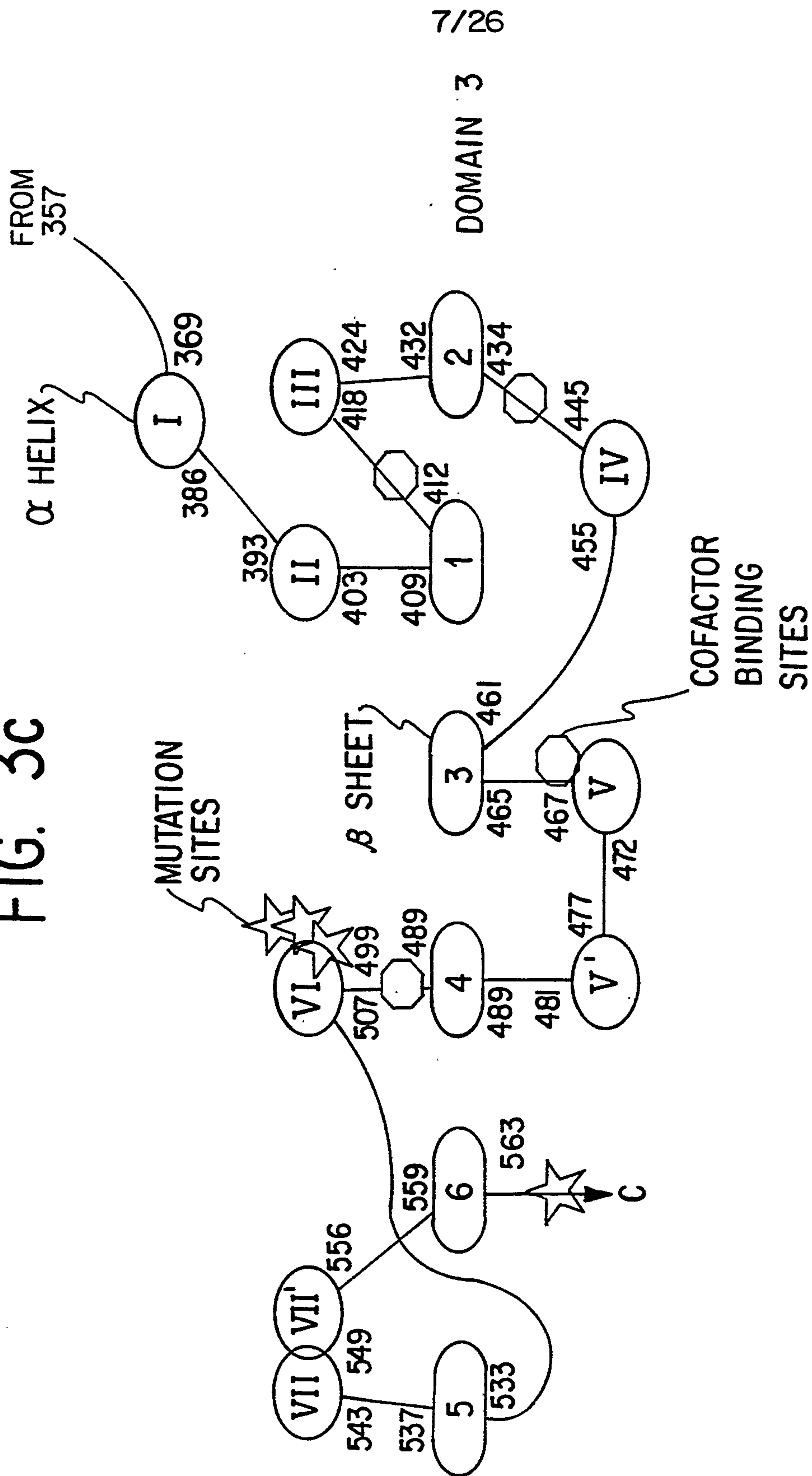
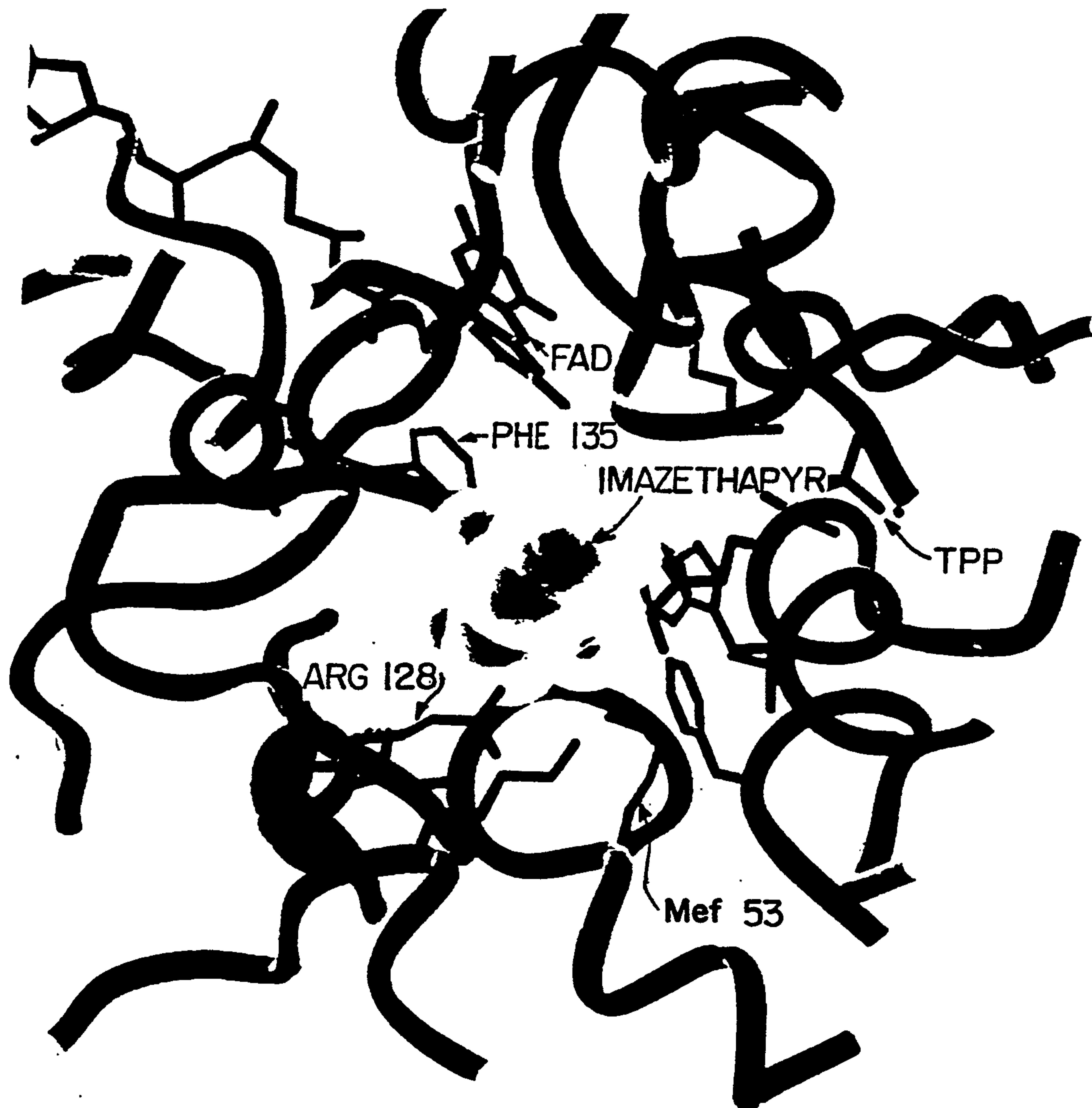


FIG. 3C



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FIG. 4



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FIG. 5D

	1	50
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P
Pac751		
Maizeals2	PKARRRAHLL	ATRRALAAPI
Maizeals1	PKSRRRAHLL	ATRRALAAPI
Tobac1	HLTHTHIIH	SQRRRTISN
Tobac2	HLTPT..HIH	SQRRRTISN
Athcsr12	RRGIKSSSPS	SISAVLNTT
Bnaal3	R.....PL	AISAVLNSPV
Bnaal2	AT	RVSVSANSKK
Consensus	--TR-RAH-L	-IRR-LN-PI
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P
Pac751		
Maizeals2		
Maizeals1		
Tobac1	MAAA...APS	SPSSAFSKTL
Tobac2	MAAA...AAA	PSPS.FSKTL
Athcsr12	MAAA'TTTT	SSSISFSTKP
Bnaal3	MAAA...TS	SSPISLTAKP
Bnaal2	M	ASFSFFGTIP
Consensus	MAAA--ATS	-S-SSFS--P</

A _____ A

FIG. 5b

A		A	
	101		150
Pac751	DILVESLERC	GVRDVFAYPG	GASMEIHQAL
Maizeals2	DILVESLERC	GVRDVFAYPG	GASMEIHQAL
Maizeals1	DILVEALERC	GVRDVFAYPG	GASMEIHQAL
Tobac1	DVLVEALERE	GVTDFVAYPG	GASMEIHQAL
Tobac2	DVLVEALERE	GVTDFVAYPG	GASMEIHQAL
Athcsr12	DILVEALERQ	GVETVFAYPG	GASMEIHQAL
Bnaal3	DILVEALERQ	GVETVFAYPG	GASMEIHQAL
Bnaal2	DILVEALERQ	GVDVVFAYPG	GASMEIHQAL
Consensus	DILVEALER-	GV-DVFAYPG	GASMEIHQAL
		↑	
	151	EQUIVALENT TO MAIZE MET 53	
Pac751	ASGYARSSGR	VGVCIAATSGP	GATNLVSALA
Maizeals2	ASGYARSSGR	VGVCIAATSGP	GATNLVSALA
Maizeals1	ASAYARSSGR	VGVCIAATSGP	GATNLVSALA
Tobac1	AEGYARATGF	PGVCIAATSGP	GATNLVSGLA
Tobac2	AEGYARATGF	PGVCIAATSGP	GATNLVSGLA
Athcsr12	AEGYARSSGK	PGICIAATSGP	GATNLVSGLA
Bnaal3	AEGYARSSGK	PGICIAATSGP	GATNLVSGLA
Bnaal2	AEGYARSSGK	PGICIAATSGP	GAMNLVSGLA
Consensus	AEGYARSSG-	PGVCIAATSGP	GATNLVSGLA
		↑	
	200	EQUIVALENT TO MAIZE ARG 128	
Pac751	ASGYARSSGR	DALLDSVPMV	AITGQVPRRM
Maizeals2	ASGYARSSGR	DALLDSVPMV	AITGQVPRRM
Maizeals1	ASAYARSSGR	DALLDSVPMV	AITGQVPRRM
Tobac1	AEGYARATGF	DALLDSVPV	AITGQVPRRM
Tobac2	AEGYARATGF	DALLDSVPV	AITGQVPRRM
Athcsr12	AEGYARSSGK	DALLDSVPLV	AITGQVPRRM
Bnaal3	AEGYARSSGK	DALLDSVPLV	AITGQVPRRM
Bnaal2	AEGYARSSGK	DALLDSVPLI	AITGQVPRRM
Consensus	AEGYARSSG-	DALLDSVP-V	AITGQVPRRM
		↑	
		EQUIVALENT TO MAIZE ARG 128	
B		B	

FIG. 5c

B_____B

EQUIVALENT TO MAIZE PHE 135

	201	↓				250
Pac751	IGTDAFQETP	IVEVTRSITK	HNVLVDVDD	IPRVVQEAF	LASSGRP	GPV
Maizeals2	IGTDAFQETP	IVEVTRSITK	HNVLVDVDD	IPRVVQEAF	LASSGRP	GPV
Maizeals1	IGTDAFQETP	IVEVTRSITK	HNVLVDVDD	IPRVVQEAF	LASSGRP	GPV
Tobac1	IGTDAFQETP	IVEVTRSITK	HNVLVMDVED	IPRVVREAF	LARSGRP	GPV
Tobac2	IGTDAFQETP	IVEVTRSITK	HNVLVMDVED	IPRVVREAF	LARSGRP	GPV
Athcsr12	IGTDAFQETP	IVEVTRSITK	HNVLVMDVED	IPRIIEEAF	LATSGRP	GPV
Bnaal3	IGTDAFQETP	IVEVTRSITK	HNVLVMDVDD	IPRIVQEAF	LATSGRP	GPV
Bnaal2	IGTMAFQETP	VVEVTRTITK	HNVLVMEVDD	IPRIVREAF	LATSVRP	GPV
Consensus	IGTDAFQETP	IVEVTRSITK	HNVLVMDVDD	IPRVVQEAF	LA-SGRP	GPV

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	251					300
Pac751	LVDIPKDIQQ	QMAVPWDKP	MSLPGYIARL	PKPPATELLE	QVLR	LVGESR
Maizeals2	LVDIPKDIQQ	QMAVPWDKP	MSLPGYIARL	PKPPATELLE	QVLR	LVGESR
Maizeals1	LVDIPKDIQQ	QMAVPAWDT	MSLPGYIARL	PKPPATEFLE	QVLR	LVGESR
Tobac1	LIDVPKDIQQ	QLVIPDWDQP	MRLPGYMSRL	PKLPNEMLLE	QIVRL	ISESK
Tobac2	LIDVPKDIQQ	QLVIPDWDQP	MRLPGYMSRL	PKLPNEMLLE	QIVRL	ISESK
Athcsr12	LVDVPKDIQQ	QLAIPNWEQA	MRLPGYMSRM	PKPPEDSHLE	QIVRL	ISESK
Bnaal3	LVDVPKDIQQ	QLAIPNWDQP	MRLPGYMSRL	PQPPEVSQLG	QIVRL	ISESK
Bnaal2	LIDVPKDVQQ	QFAIPNWEQP	MRLPLYMSTM	PKPPKVSHLE	QILRL	VSESK
Consensus	LVDVPKDIQQ	QLAIPNWDQP	MRLPGYMSRL	PKPPA--LLE	QI-RL-	SESK

C_____C

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	301					350
Pac751	RPVLYVGGGC	ARSGEELRRF	VELTGIPVTT	TLMGLGNFPS	DD	PLSLRML
Maizeals2	RPVLYVGGGC	AASGEELRRF	VELTGIPVTT	TLMGLGNFPS	DD	PLSLRML
Maizeals1	RPVLYVGGGC	AASGEELCRF	VELTGIPVTT	TLMGLGNFPS	DD	PLSLRML
Tobac1	KPVLYVGGGC	SQSSEDLRRF	VELTGIPVAS	TLMGLGAFPT	GD	ELSLSML
Tobac2	KPVLYVGGGC	SQSSEELRRF	VELTGIPVAS	TLMGLGAFPT	GD	ELSLSML
Athcsr12	KPVLYVGGGC	LNSSEELGRF	VELTGIPVAS	TLMGLGSYPC	DD	ELSLHML
Bnaal3	RPVLYVGGGS	LNSSEELGRF	VELTGIPVAS	TLMGLGSYPC	ND	ELSLQML
Bnaal2	RPVLYVGGGC	LNSSEELRRF	VELTGIPVAS	TFMGLGSYPC	DDEE	FSLQML
Consensus	RPVLYVGGGC	-NSSEELRRF	VELTGIPVAS	TLMGLG-FP-	DD	ELSLRML

	351			400		
Pac751	GMHGT	VYANY	AVDKADLLLA	LGVRFDDRV	GKIEAFASRA	KIVHVDIDPA
Maize1s2	GMHGT	VYANY	AVDKADLLLA	LGVRFDDRV	GKIEAFASRA	KIVHVDIDPA
Maize1s1	GMHGT	VYANY	AVDKADLLLA	FGVRFDDRV	GKIEAFAGRA	KIVHIDIDPA
Tobac1	GMHGT	VYANY	AVDSSDLLLA	FGVRFDDRV	GKLEAFASRA	KIVHIDIDSA
Tobac2	GMHGT	VYANY	AVDSSDLLLA	FGVRFDDRV	GKLEAFASRA	KIVHIDIDSA
Athcsr12	GMHGT	VYANY	AVEHSDLLLA	FGVRFDDRV	GKLEAFASRA	KIVHIDIDSA
Bnaal3	GMHGT	VYANY	AVEHSDLLLA	FGVRFDDRV	GKLEAFASRA	KIVHIDIDSA
Bnaal2	GMHGT	VYANY	AVEYSDLLLA	FGVRFDDRV	GKLEAFASRA	KIVHIDIDST
Consensus	GMHGT	VYANY	AVDKSDLLLA	FGVRFDDRV	GKLEAFASRA	KIVHIDIDSA

D

FIG. 5e

—

	401				450
Pac751	EIGKNKQPHV	SICADVKLAL	QGMNALLEGS	TSKKSFDGGS	WNDELDQQKR
Maizeals2	EIGKNKQPHV	SICADVKLAL	QGMNALLEGS	TSKKSFDGGS	WNDELDQQKR
Maizeals1	EIGKNKQPHV	SICADVKLAL	QGMNTLLEGS	TSKKSFDGGS	WHDELDQQKR
Tobac1	EIGKNKQPHV	SICADIKLAL	QGLNSILESK	EGKLKLDGSA	WRQELTEQKV
Tobac2	EIGKNKQPHV	SICADIKLAL	QGLNSILESK	EGKLKLDGSA	WRQELTVQKV
Athcsr12	EIGKNKTPHV	SVCGDVKLAL	QGMNKVLENR	AEELKLDGCV	WRNELNVQKQ
Bnaal3	EIGKNKTPHV	SVCGDVKLAL	QGMNKVLENR	AEELKLDGCV	WRSELSEQKQ
Bnaal2	EIGKNKTPHV	SVCCDVQLAL	QGMNEVLENR	RD. . VLDGGE	WRCELNEQRL
Consensus	EIGKNKQPHV	SICADVKLAL	QGMN-VLE--	T-KLKLDGGS	WRDELD-QKR

	451			500	
Pac751	EFPLGYKTSN	EEIQPQYAIQ	VLDELTKGEA	IIGTGVGQHQ	MWAAQYYTYK
Maizeals2	EFPLGYKTSN	EEIQPQYAIQ	VLDELTKGEA	IIGTGVGQHQ	MWAAQYYTYK
Maizeals1	EFPLGYKIFN	EEIQPQYAIQ	VLDELTKGEA	IIATGVGQHQ	MWAAQYYTYK
Tobac1	KHPLNFKTFG	DAIPPQYAIQ	VLDELTINGNA	IISTGVGQHQ	MWAAQYYKYR
Tobac2	KYPLNFKTFG	DAIPPQYAIQ	VLDELTINGSA	IISTGVGQHQ	MWAAQYYKYR
Athcsr12	KFPLSFKTFG	EAIPPQYAIK	VLDELTDGKA	IISTGVGQHQ	MWAAQFYNYK
Bnaal3	KFPLSFKTFG	EAIPPQYAIQ	VLDELTDGKA	IISTGVGQHQ	MWAAQFYKYR
Bnaal2	KFPLRYKTFG	EEIPPQYAIQ	LLDELTDGKA	IITGVGQHQ	MWAAQFYRFX
Consensus	KFPLG-KTFG	E-IPPQYAIQ	VLDELTKG-A	IISTGVGQHQ	MWAAQYY-YK

FIG. 5f

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E_____E

	501			550	
Pac751	RPRQLSSAG	LGAMGFGLPA	AAGASVANPG	VTVVDIDGDG	SFLMNVQELA
Maizeals2	RPRQLSSAG	LGAMGFGLPA	AAGASVANPG	VTVVDIDGDG	SFLMNVQELA
Maizeals1	RPRQLSSAG	LGAMGFGLPA	AAGAAVANPG	VTVVDIDGDG	SFLMNIQELA
Tobac1	KPRQLTSGG	LGAMGFGLPA	AIGAAVGRPD	EVVVDIDGDG	SFIMNVQELA
Tobac2	KPRQLTSGG	LGAMGFGLPA	AIGAAVGRPD	EVVVDIDGDG	SFIMNVQELA
Athcsr12	KPRQLSSGG	LGAMGFGLPA	AIGASVANPD	AIVVDIDGDG	SFIMNVQELA
Bnaal3	KPRQLSSGG	LGAMGFGLPA	AIGASVANPD	AIVVDIDGDG	SFIMNVQELA
Bnaal2	KPRQLSSGG	LGAMGFGLPA	AMGAAIANPG	AVVVDIDGDG	SFIMNIQELA
Consensus	KPRQLSSGG	LGAMGFGLPA	AIGA - VANP -	- VVVDIDGDG	SFIMNVQELA

	551			600	
Pac751	MIRIENLPVK	VFVLNNQHLG	MVVQWEDRFY	KANRAHTYLG	NPENESEIYP
Maizeals2	MIRIENLPVK	VFVLNNQHLG	MVVQWEDRFY	KANRAHTYLG	NPENESEIYP
Maizeals1	MIRIENLPVK	VFVLNNQHLG	MVVQWEDRFY	KANRAHTFLG	NPENESEIYP
Tobac1	TIKVENLPVK	IMLLNNQHLG	MVVQWEDRFY	KANRAHTYLG	NPSNEAEIYP
Tobac2	TIKVENLPVK	IMLLNNQHLG	MVVQWEDRFY	KANRAHTYLG	NPSNEAEIYP
Athcsr12	TIRVENLPVK	VLLNNQHLG	MVMQWEDRFY	KANRAHTFLG	DPAQEDEIYP
Bnaal3	TIRVENLPVK	ILLNNQHLG	MVMQWEDRFY	KANRAHTYLG	DPARENEIYP
Bnaal2	TIRVENLPVK	VLLINNQHLG	MVLQWEDHFF	AANRADSFGL	DPANPEAVFP
Consensus	TIRVENLPVK	V - LLNNQHLG	MVVQWEDRFY	KANRAHTYLG	NP - NESEIYP

F_____F

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FIG. 5g

F		F	
			650
Pac751	DFVTIAKGFN	IPAVRVTKKN	EVRAAIKKML
Maizeals2	DFVTIAKGFN	IPAVRVTKKN	EVRAAIKKML
Maizeals1	DFVAIAKGFN	IPAVRVTKKS	EVHAAIKKML
Tobac1	NMLKFAEACG	VPAARVTHRD	DLRAAIQKML
Tobac2	NMLKFAEACG	VPAARVTHRD	DLRAAIQKML
Athcsr12	NMLLFAAACG	IPAAARVTKKA	DLREAIQTML
Bnaal3	NMLQFAGACG	IPAAARVTKKE	ELREAIQTML
Bnaal2	DMLLFAASCG	IPAAARVTRRE	DLREAIQTML
Consensus	-ML-FAKACG	IPAAARVTKK-	-LRAAIQKML
			673
Pac751	MIPSGGAFKD	MILDGDGRTV	Y..
Maizeals2	MIPSGGAFKD	MILDGDGRTV	Y*.
Maizeals1	MIPSGGAFKD	MILDGDGRTV	Y*.
Tobac1	MIPSGGAFKD	VITEGDGRSS	Y*.
Tobac2	MIPSGGAFKD	VITEGDGRSS	Y*.
Athcsr12	MIPNGGTFND	VITEGDGRIK	Y*E
Bnaal3	MIPSGGTFKD	VITEGDGRTK	Y*.
Bnaal2	LIPSGGTFKD	IIV*.....	...
Consensus	MIPSGGAFKD	VITEGDGRTV	Y--
			G

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FIG. 5h

G _____ G

Pac751 - maize als2 AHAS isozyme as expressed from the pAC751 E. coli

expression vector (same as figure 1)

Maizeals2 - maize als2 AHAS isozyme (plant)

Maizeals1 - maize als1 AHAS isozyme (plant)

Tobac1 - tobacco AHAS SuRA isozyme (plant)

Tobac2 - tobacco AHAS SuRB isozyme (plant)

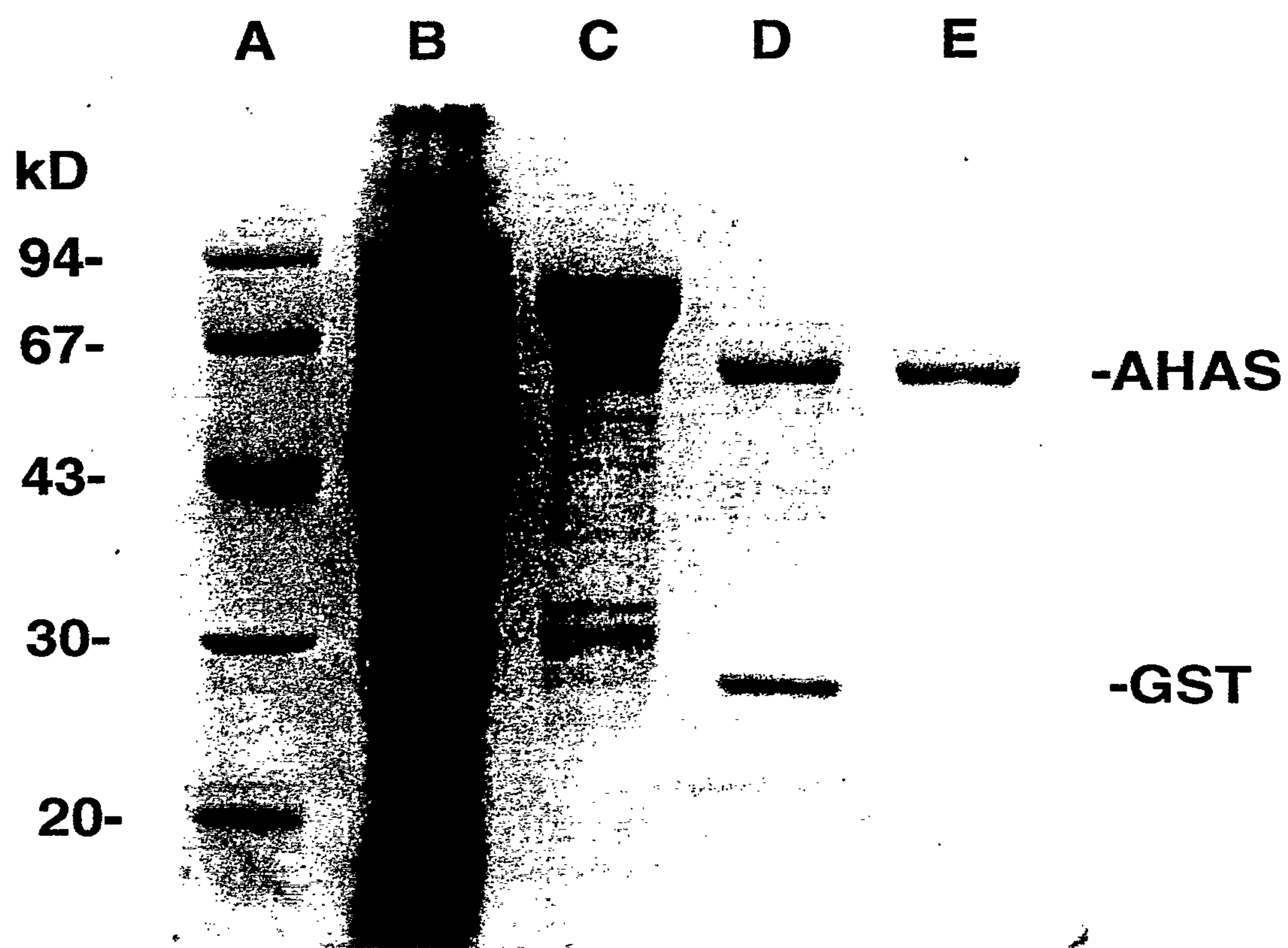
Athcsr12 - Arabidopsis thaliana Csr 1.2 AHAS gene (plant)

Bnaal3 - Brassica napus AHAS III isozyme (plant)

Bnaal2 - Brassica napus AHAS II isozyme (plant)

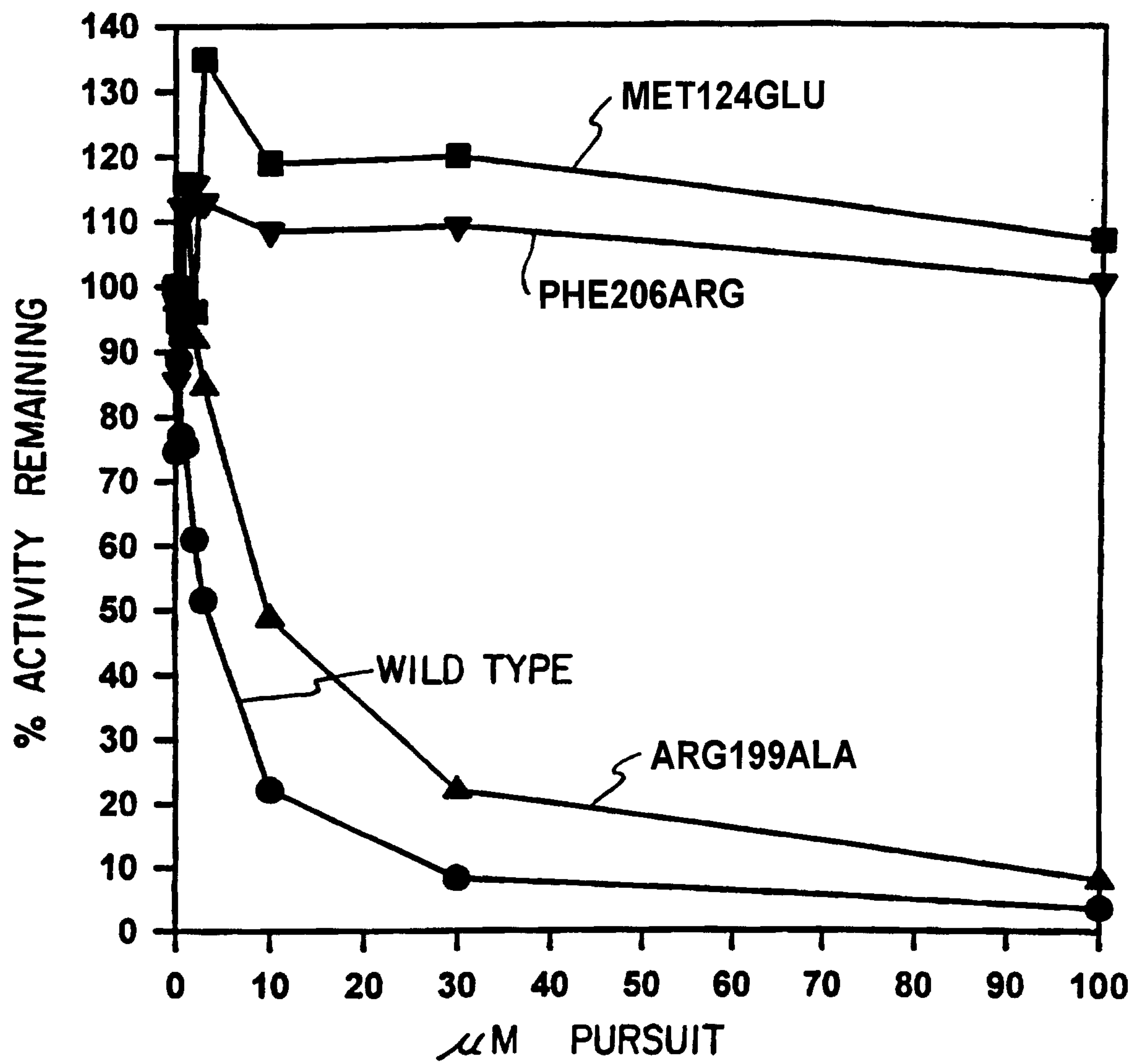
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FIG. 6



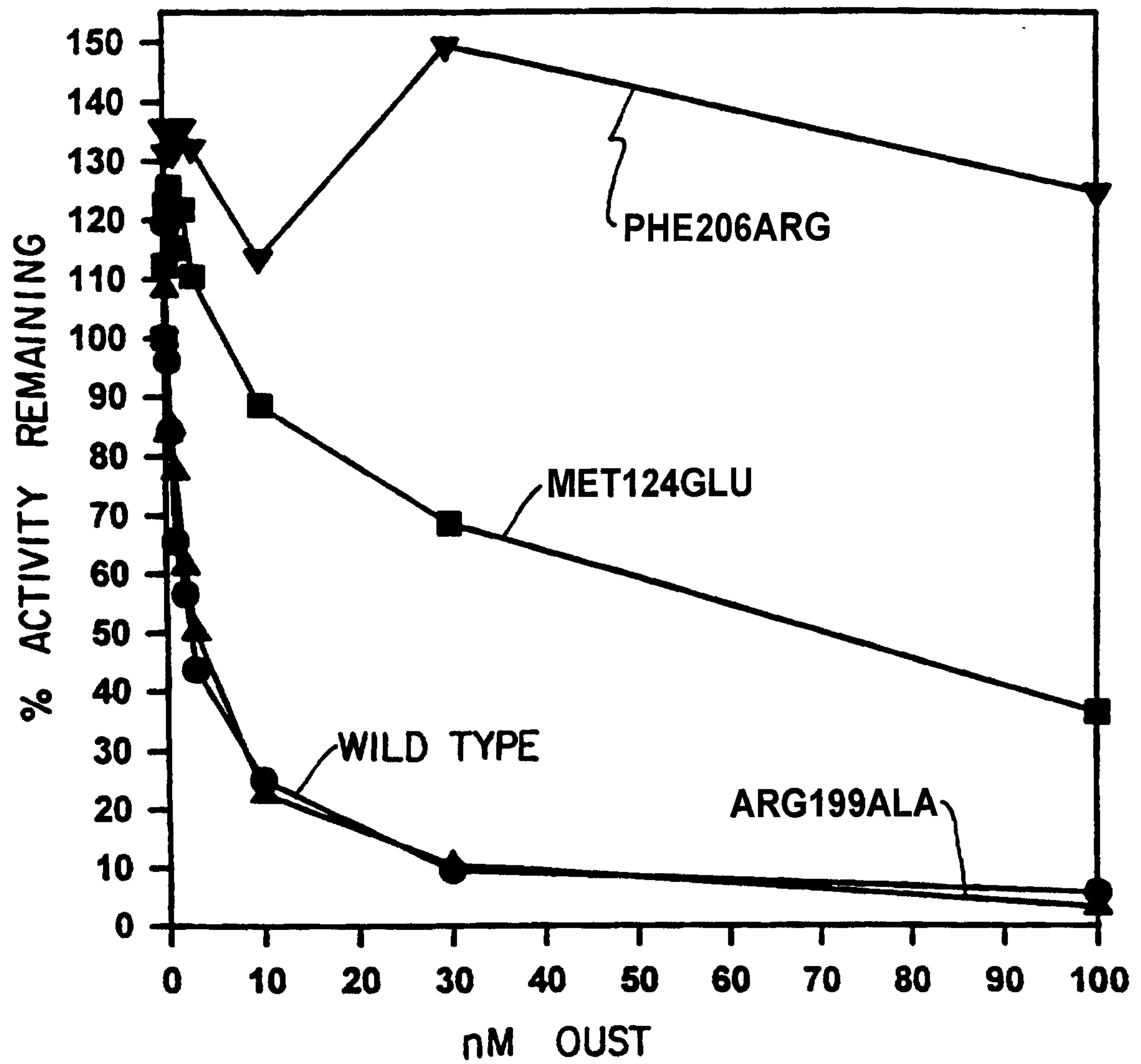
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FIG. 7



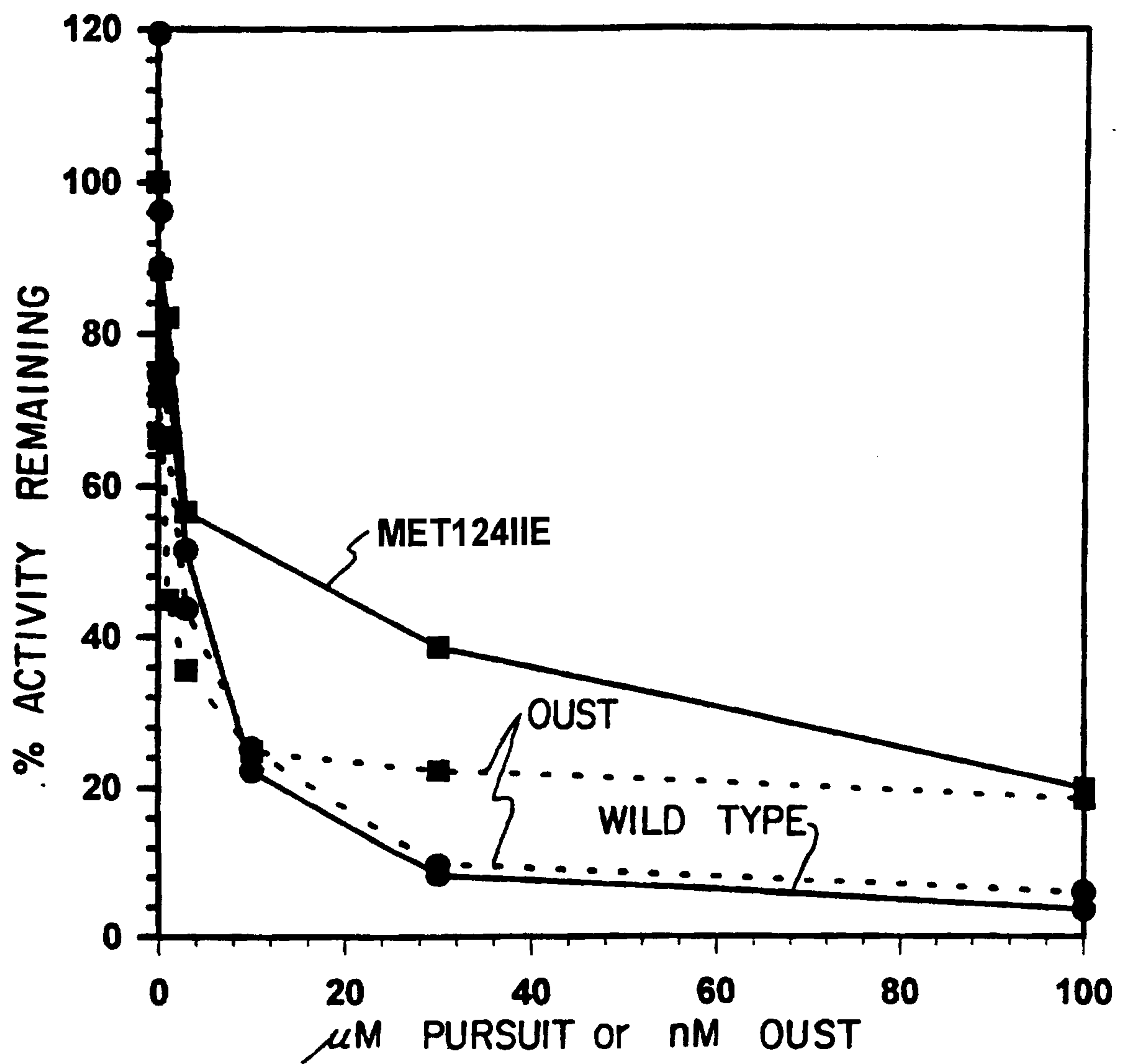
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FIG. 8



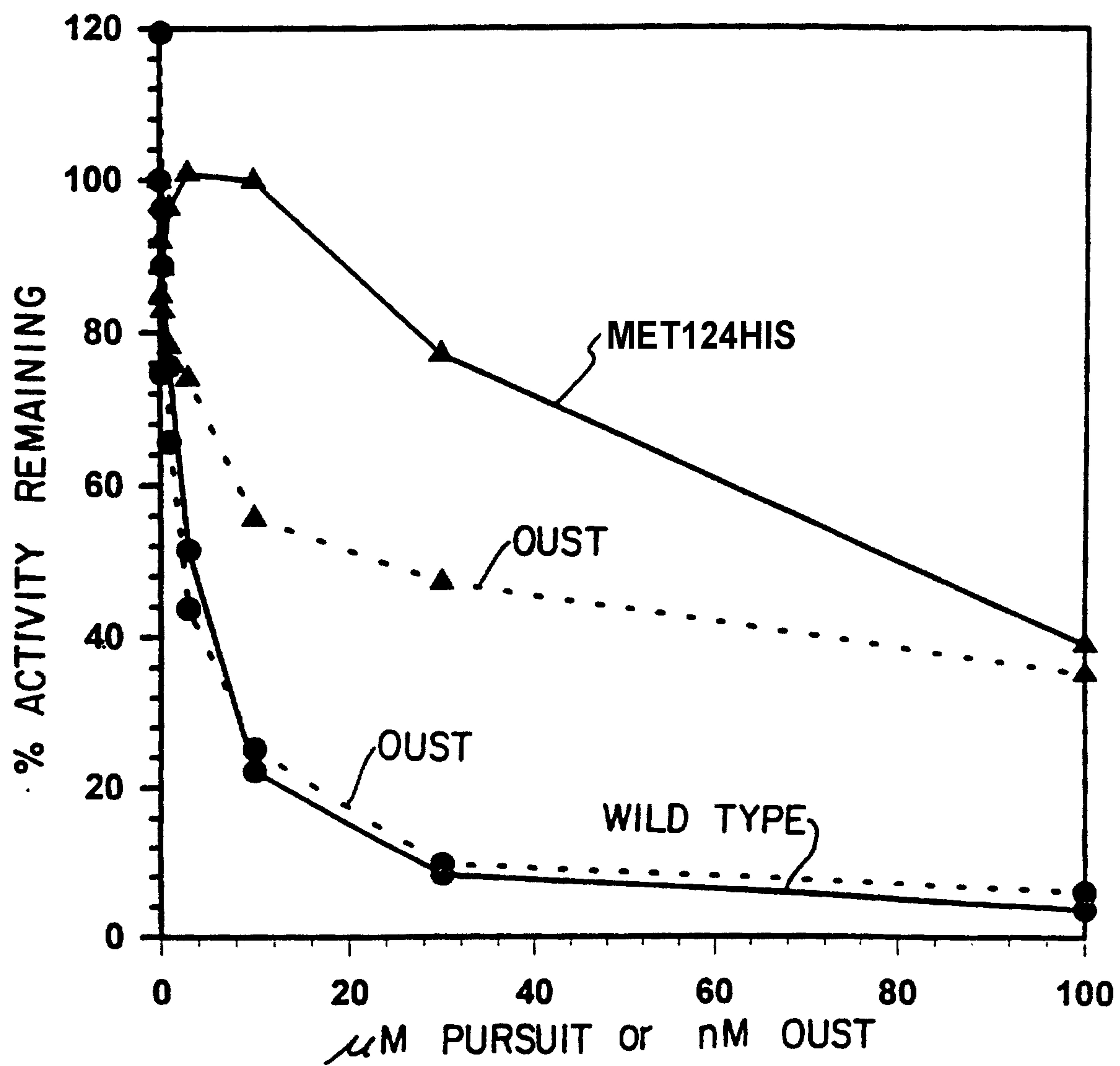
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FIG. 9



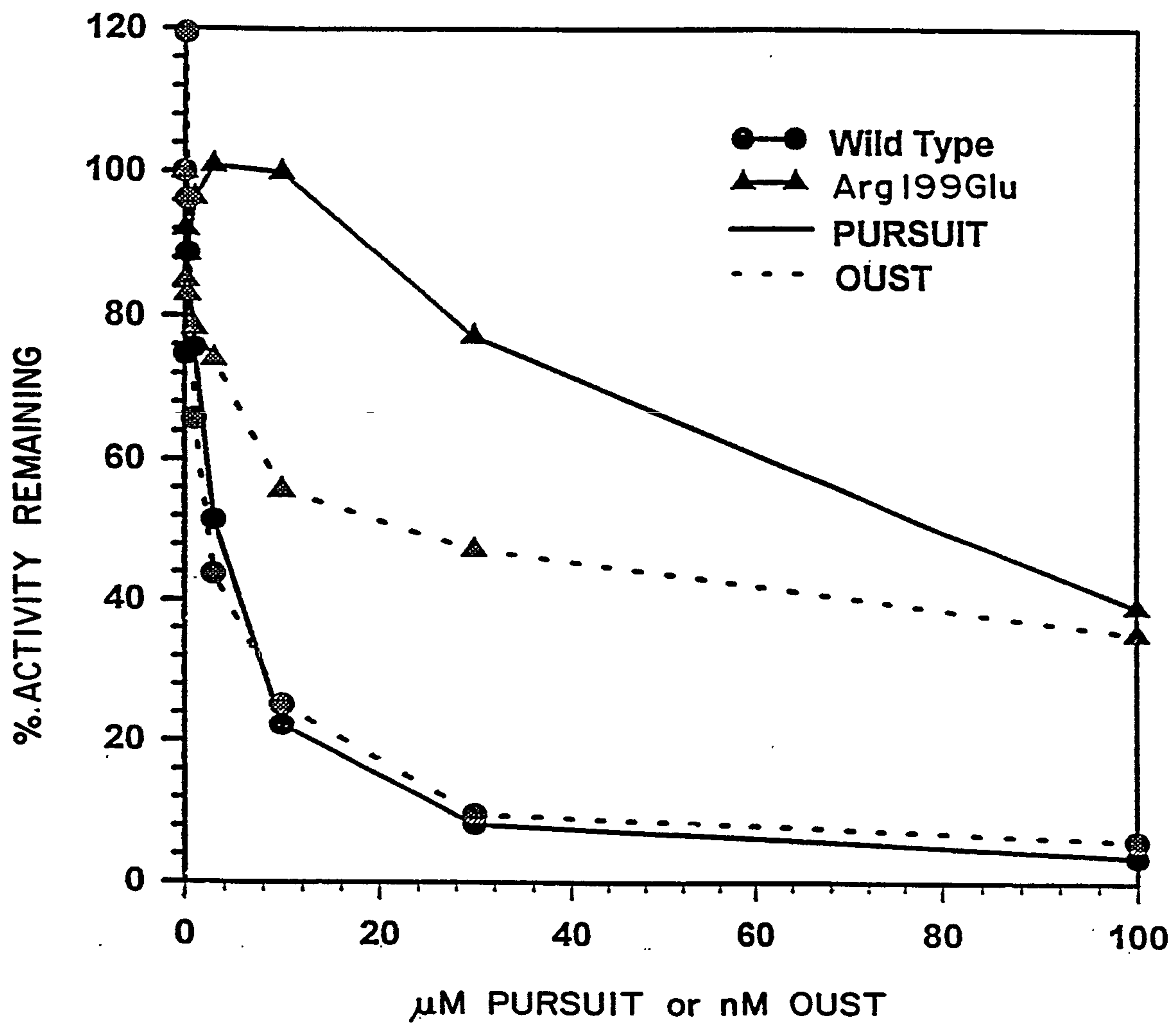
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FIG. 10



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FIG. 11



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FIG. 12

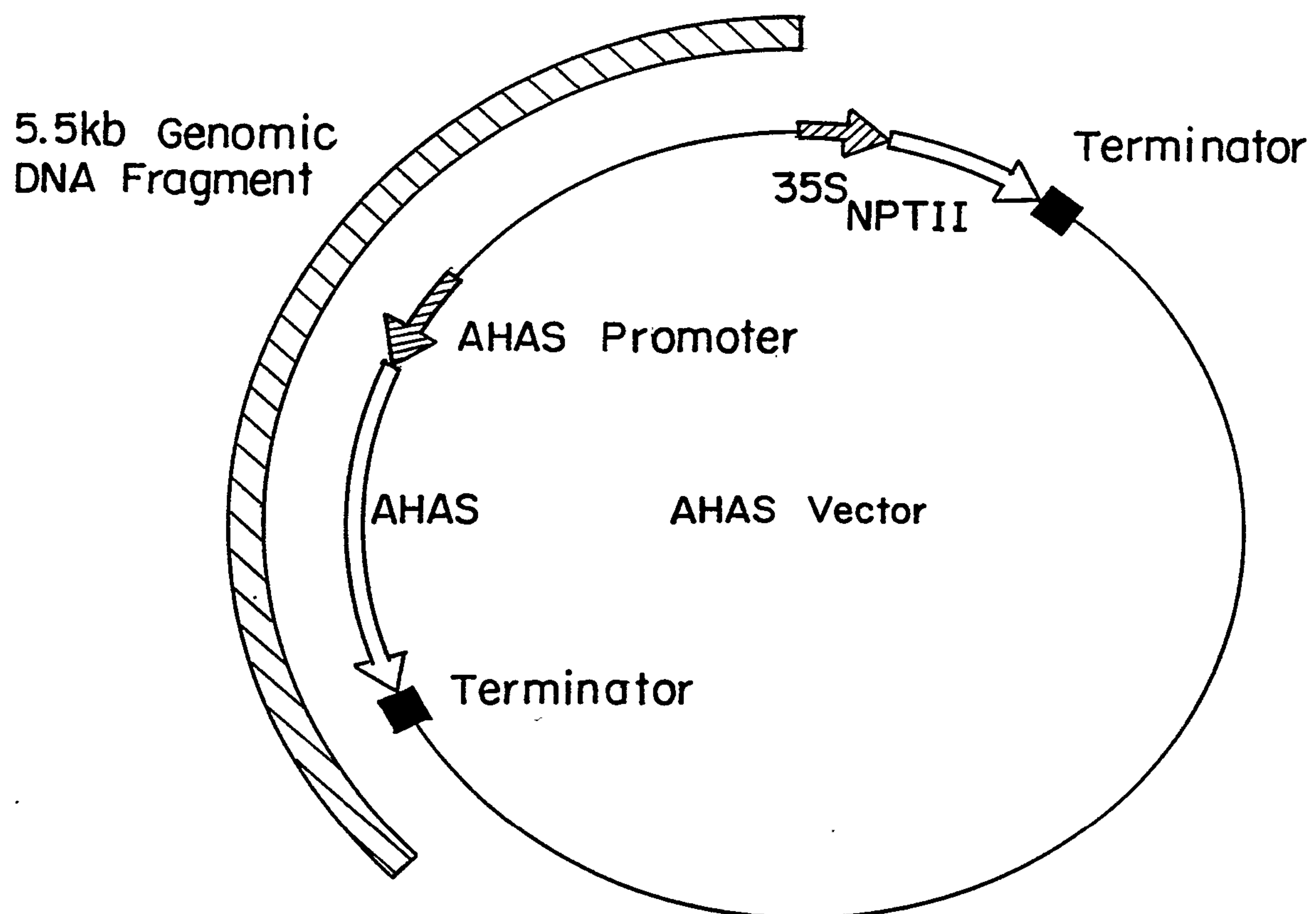


FIG. 13

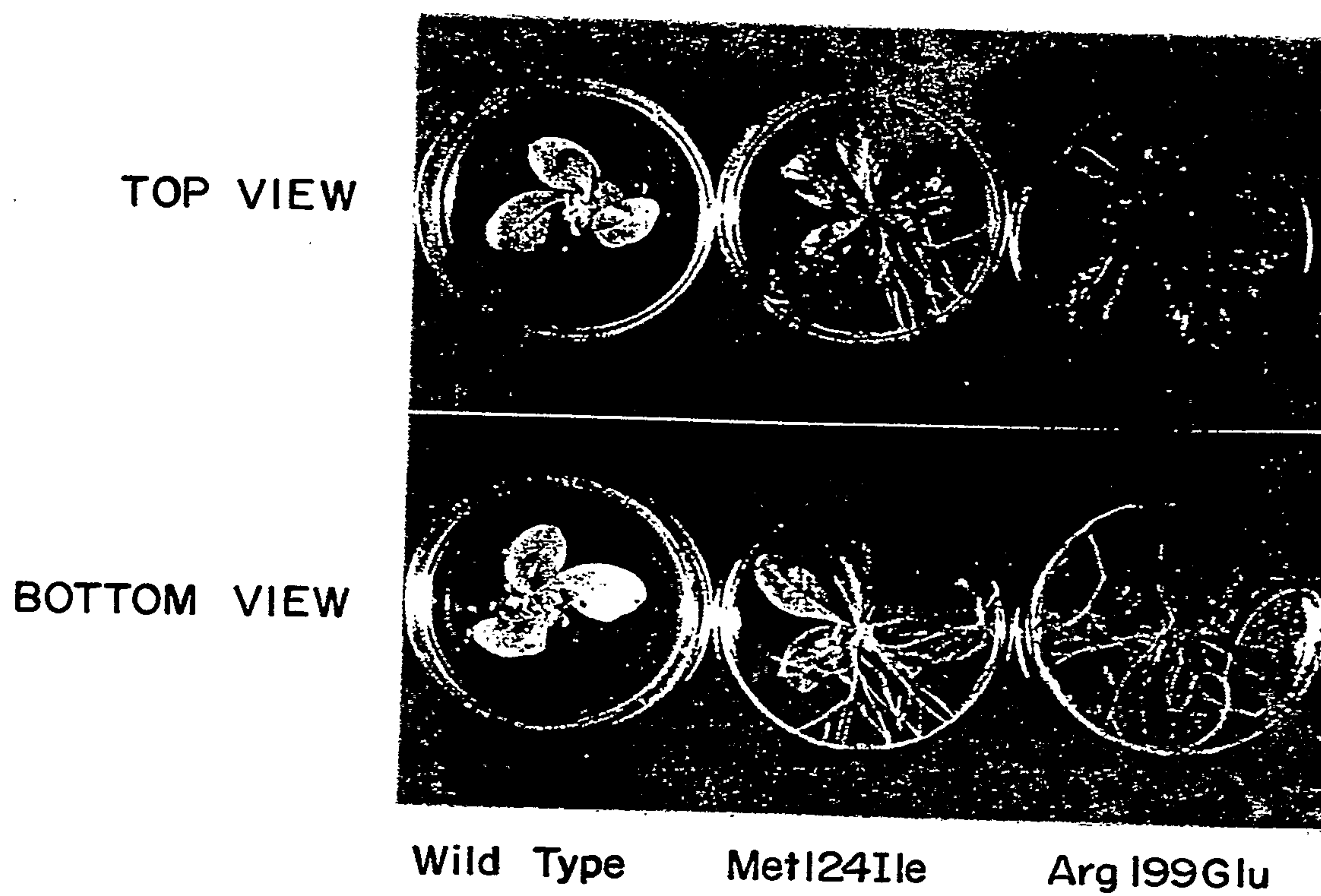
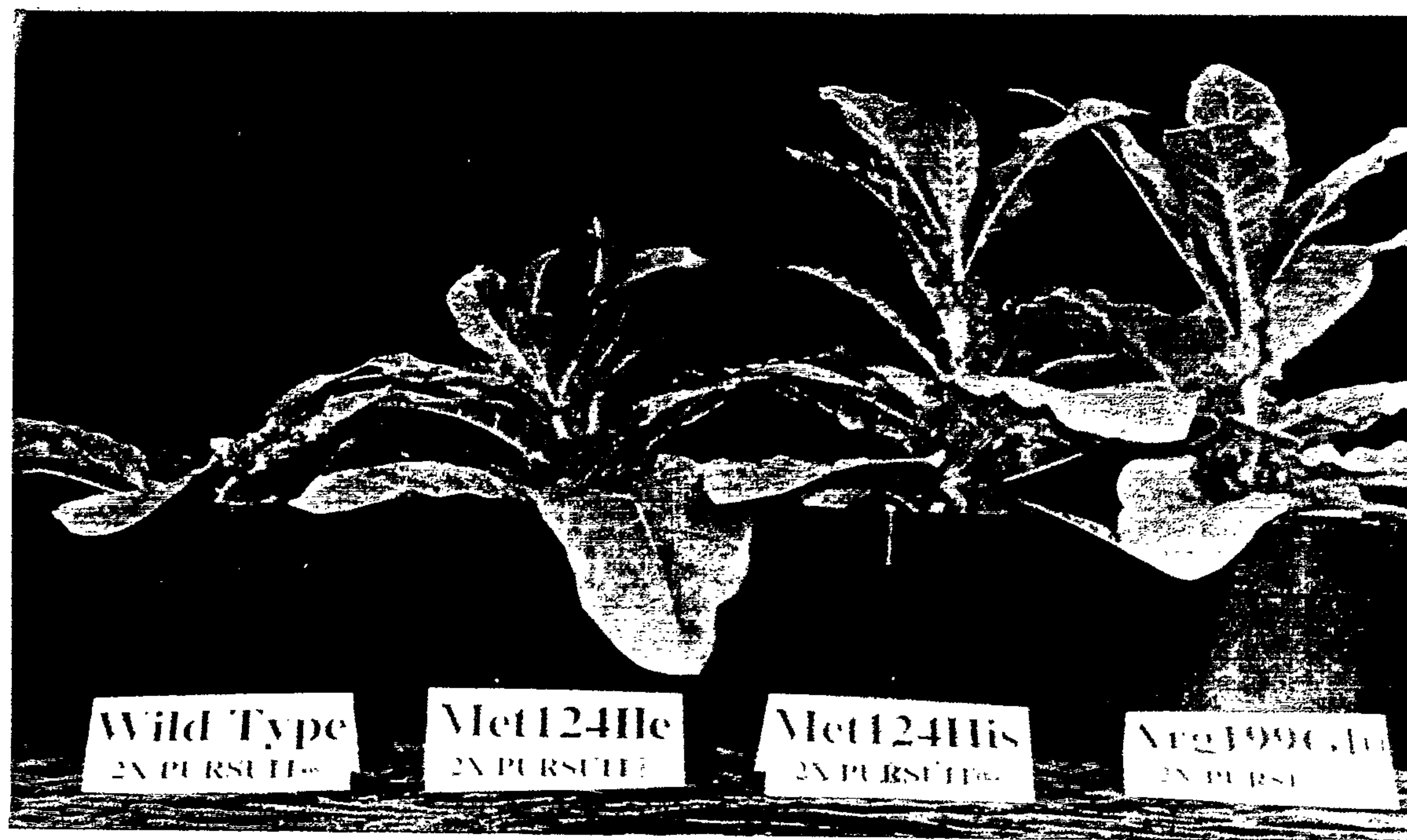
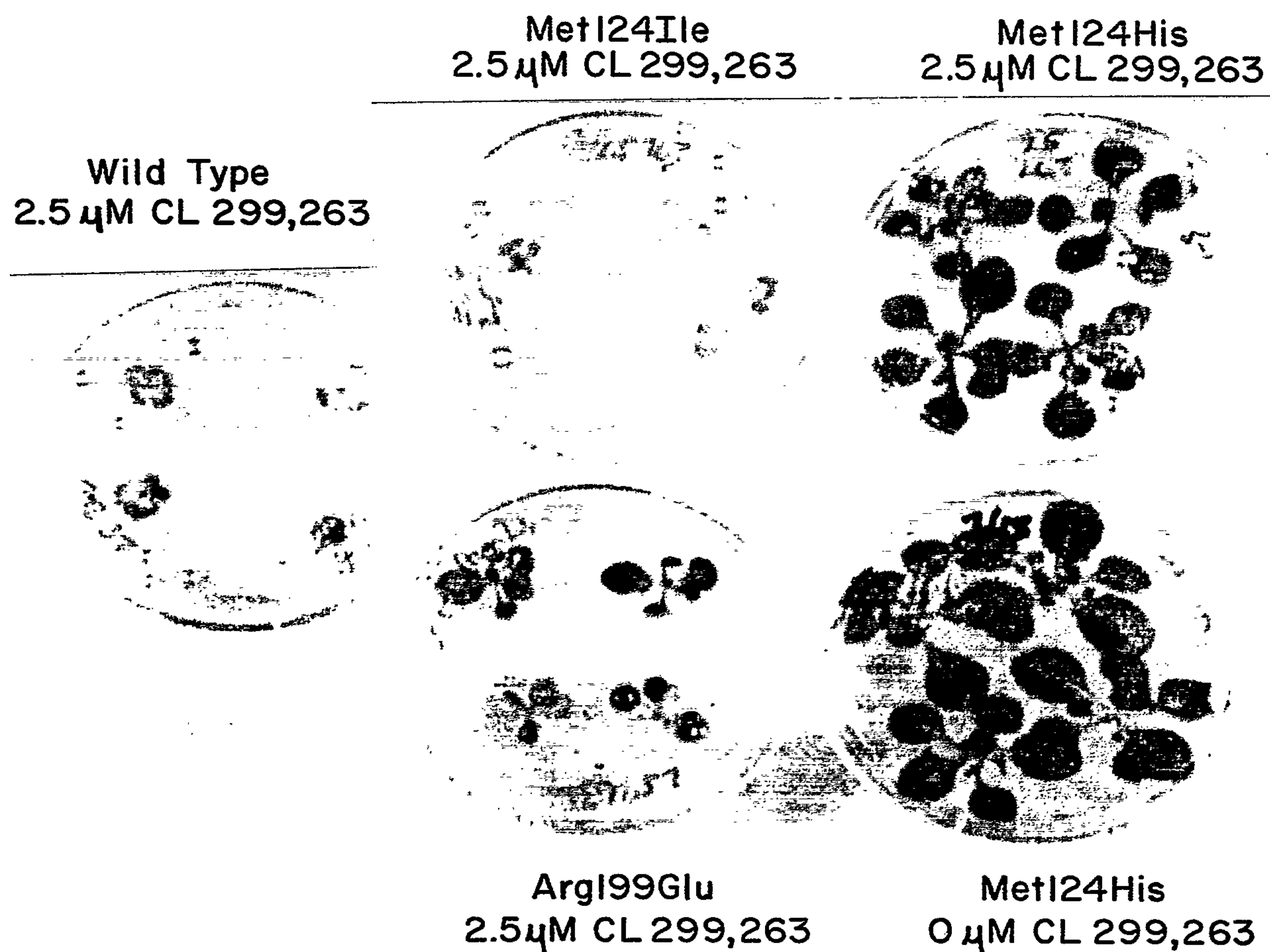


FIG. 14



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FIG. 15



% ACTIVITY REMAINING

