Position	Change	Basis*	Position	Change	Basis*	Position	Change	Basis*
849	C>G	1	1458	C>G	1	1834-36	CCA	7
852	C>T	3, 4, 9	1465	A>T	3	1837-39	TAG	8
861	C>T	1	1466	G>C	3			
921	C>G	1	1467	C>G	1			
927	C>T	3, 4, 9						

*BASIS CODES: 1=Eliminate CG doublet; 2=Create Sal I site; 3=Generate codon diversity; 4=Reduce G+C content; 5=Create Kpn I site; 6=Eliminate Sal I site; 7= Proline codon; 8=Stop codon; 9=Generate CT doublet; 10=Eliminate Nar I site.

Two proline codons and a stop codon (TAG) were added to the end of the sequence (total amino acids now about 612), thereby producing MZE HD73 #4 trnc+. The presence of terminal proline residues is thought to reduce carboxy-terminus proteolysis. The resulting sequence was scanned for restriction sites. Base changes were made to eliminate a Sal I site at position 1219, create a new one at position 181, eliminate a Nar I site at position 158, and create a new Kpn I site at position 1217. An ORF search revealed the ICP ORF in frame 1, and one small ORF each in frames 2 and 3. The long frame 3 ORF present in previous versions of the gene was interrupted by a stop codon at base 78; no other ORFS beginning with an ATG and longer than 25 amino acids was present in frame 3.

Mze HD73 #5 trnc+: Reduction of GC content and increase in codon diversity. Comparison of base doublet frequencies between versions #4 trnc+ and previous versions of the sequence (Table 3) revealed that the base composition had been altered towards reductions in CG base pairs, and towards abundances in TG and CT base pairs. However, version #4 trnc+ still had a relatively high G+C content (62%) compared to the target of 55-60% for maize genes. Reduction of this parameter necessitated using more alternate codons containing A and/or T.

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Table 8 summarizes the changes made to the sequence of Mze HD73 #4 trnc+ to generate Mze HD73 #5 trnc+.

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Table 8. Changes made in Mze HD73 #4 trnc+ → Mze HD73 #5 trnc+.

Position	Change	Basis	Position	Change	Basis	Position	Change	Basis
15	C>A	1	660	C>T	1	1149	G>T	1
36	C>T	1	667	A>T	2	1177	A>T	1, 2
46	C>T	1	668	G>C	2	1178	G>C	1, 2
57	C>T	1	684	C>T	1	1179	C>T	1, 2
72	G>T	1	693	G>A	1	1186	C>T	1
87	C>T	1	723	G>T	1	1191	C>A	1
105	C>A	1	735	C>T	1	1197	C>T	1
115	A>T	1, 2	744	C>A	1	1228	C>T	1
116	G>C	1, 2	754	A>T	2	1242	C>T	1
117	C>T	1, 2	755	G>C	2	1245	C>A	1
132	G>A	1	759	C>T	1	1263	C>A	1
142	A>T	1, 2	768	C>A	1	1266	C>T	1
143	G>C	1, 2	781	A>T	1, 2	1272	G>A	1
144	C>T	1, 2	782	G>C	1, 2	1279	A>T	1, 2
174	G>T	1	783	C>T	1, 2	1280	G>C	1, 2
177	C>T	1	792	C>T	1	1281	C>T	1, 2
210	C>A	1	816	G>T	1	1288	C>T	1
216	G>A	1	817	C>T	1	1299	G>T	1
225	C>T	1	831	C>T	1	1300	A>T	2
231	G>T	1	835	A>T	2	1301	G>C	2
237	G>A	1	836	G>C	2	1311	C>T	1
258	G>A	1	849	G>T	1	1341	C>T	1
276	C>T	1	855	G>A	1	1360	A>T	2
285	G>A	1	873	C>T	1	1361	G>C	2
292	A>T	2	877	A>T	1, 2	1374	C>T	1
293	G>C	2	878	G>C	1, 2	1416	C>T	1
300	G>T	1	879	C>T	1, 2	1419	G>A	1
307	C>T	1	882	C>T	1	1425	C>T	1
363	C>A	1	897	C>T	1	1431	G>T	1
376	C>T	1	900	G>T	1	1446	G>T	1
399	G>A	1	912	C>T	1	1494	G>T	1
415	A>T	1, 2	960	G>A	1	1501	C>T	1
416	G>C	1, 2	969	C>T	1	1536	C>T	1

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417	C>T	1, 2	978	G>T	1	1551	C>A	1
420	C>T	1	981	C>T	1	1563	C>A	1
438	C>T	1	1008	C>T	1	1564	A>T	2
462	G>A	1	1009	C>T	1	1565	G>C	2
468	C>T	1	1017	C>T	1	1614	C>A	1
471	G>T	1	1035	C>T	1	1621	C>T	1
489	G>A	1	1038	C>A	1	1645	A>T	2
499	C>T	1	1041	G>A	1	1646	G>C	2
510	G>T	1	1056	C>T	1	1686	G>T	1
514	C>T	1	1059	G>A	1	1722	G>A	1
526	A>T	1, 2	1068	G>A	1	1734	C>T	1
527	G>C	1, 2	1086	G>T	1	1744	A>T	1, 2
528	C>T	1, 2	1087	A>T	2	1745	G>C	1, 2
537	C>T	1	1088	G>C	2	1746	C>T	1, 2
540	G>A	1	1096	C>T	1	1749	G>T	1
571	A>T	1, 2	1110	C>A	1	1812	G>A	1, 3
572	G>C	1, 2	1135	G>A	1	1830	C>T	1
573	C>T	1, 2	1140	G>T	1			
621	C>T	1	1143	G>T				

*BASIS CODES: 1=Reduce G+C content; 2=Generate codon diversity; 3=Create EcoR I site.

As shown by the Basis Codes for the table, these changes were made to reduce the G+C content of the DNA and to introduce additional codon diversity, without sacrificing an inordinant amount of codon bias. Where possible, blocks of high G+C sequence were interrupted by the addition of T or A substitutions. Also, a unique EcoR I site was created near the 3' end of the gene to provide for possible future sequence additions. Substitute codons choices useful to reduce GC content are set forth in Table 9.

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Table 9. Alternative codons used to reduce G+C content or increase CT or TG doublets.

	Substitute Codon Choices[a]			
Amino Acid	Preferred	1st Alternate	2nd Alternate	3rd Alternate
Ala	GCC (36)	GCT (27)	GCG (24)	
Arg	CGC (40)	AGG (26)	CGG (13)	CGT (11)
Asp	GAC (76)	GAT (24)		
Gln	CAG (59)	CAA (41)		
Glu	GAG (81)	GAA (19)		
Gly	GGC (50)	GGT (21)	GGG (16)	
Ile	ATC (68)	ATT (24)		
Leu	CTG (31)	CTC (28)	CTT (13)	TTG (13)
Pro	CCC (30)	CCG (27)	CCA (23)	CCT (20)
Ser	AGC (28)	TCC (27)	TCG (16)	TCT (14)
Thr	ACC (47)	ACG (26)	ACT (16)	
Val	GTG (40)	GTRC(37)	GTT (17)	

[a] Numbers in parentheses are frequencies of usage in maize genes (from Table 2).

Substitutions (listed in Table 9) were made with the following rationales listed below:

i) While all Pro codons are acceptable substitutes for one another, CCT generates a CT doublet, as well as lowers the G+C content.

ii) The two Gln codons are present in maize genes in approximately equal frequencies, and can therefore be readily substituted for one another. Similarly, the Ser codons AGC and TCC are considered to be interchangeable. Analogous frequency similarities exist for the Val codons GTG and GTC, Leu codons CTG and CTC, and Ala minor codons GCT and GCG.

iii) Leu and Ser minor codons TTG and TCT are acceptable when they follow a C-ending codon, so that additional CT doublets are generated. TTG offers the added feature of increasing the TG doublet count.

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iv) Arg codon AGG may be substituted for the preferred codon CGC (see discussion in previous section). Although AGG occurs in maize genes at substantially lower frequency than the preferred codon, it is found twice as often as the third choice codon.

v) Minor codons such as GAT (Asp), GAA (Glu), ATT (Ile), ACT (Thr), and GTT (Val), which have obviously been counterselected in maize, should be used sparingly, if possible. It is preferable that they be placed before or after codons that will contribute to the formation of a CT or TG doublet. Because they are a feature of native maize genes, their inclusion in a synthetic gene need not be avoided entirely.

Mze HD73 #6 trnc+. Only a few changes were made to the sequence of Mze HD73 #5 trnc+ to generate the final version of the gene, Mze HD73 #6 trnc+. (summarized in Table 10).

Table 10. Mze HD73 #5 trnc+ → Mze HD73 #6 trnc+.

Position	Change	Rationale
1831-33	CCA → ACC	Pro → Thr
1834-36	CCA → CTG	Pro → Leu
1837-39	TAG → GAG	Stop → Glu
1840-42	GCT	Ala codon
1843-45	GAG	Glu codon
1846-48	CCA	Pro codon
1849-51	CCA	Pro codon
1852-54	TAG	Stop codon

As summarized in Table 11, changes resulting in Mze HD73 #5 trnc+ and Mze HD73 #6 trnc+ diminished the numbers of CG doublets by almost 50%, and clearly enriched in TG and CT doublets. Moreover, the G+C content of 56% falls well within the range of maize metabolic genes.

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Table 11. Comparisons of base doublet numbers and base compositions of ICP genes.

	Truncated HD73 Gene Versions						
Doublet	BT 1830 bp	Mze #1 1830 bp	Mze #2 1830 bp	Mze #3 1830 bp	Mze #4 1836 bp[a]	Mze #5 1836 bp[a]	Mze #6 1851 bp[a]
TA	174	27	27	27	29[a]	36[a]	37[a]
CG	46	171	170	168	102	90	90
TG	102	110	107	105	138	142	144
CT	86	98	98	98	129	165	167
% G+C	37	66	66	66	62	56	56

[a] TAG stop codon, not considered to be part of the coding region, is ignored.

Examination of the DNA sequence that Perlak et al. (PNAS, 88 (1991) 3324) successfully expressed in transgenic plants revealed that the gene encoded 615 amino acids of the native ICP (rather than the 610 encoded by MZE HD73 #5 trnc+). Codons for the five (5) additional amino acids were therefore added between codon 610 and the two (2) Pro codons added in version #4. MZE HD73 #6 trnc+ therefore encodes 615 amino acids of native HD73 ICP, and two carboxy-terminus proline residues (SEQ. ID. NO. 1).

Table 12 below lists the codon usage patterns of the native *Bacillus* HD73 gene, the Mze HD73 #1 trnc+ gene, and Mze HD73 #6 trnc+.

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Table 12. Comparisons of codon numbers of ICP genes.

Amino Acid	Codon[a]	Bt	Mze # 1	Mze # 6	Amino Acid	Codon[a]	Bt	Mze # 1	Mze # 6
Gly	GGG(16)	8	0	0	Ile	ATA(8)	18	0	0
Gly	GGA(13)	19	0	0	Ile	ATT(24)	23	0	13
Gly	GGT(21	13	0	18	Ile	ATC(68)	6	47	34
Gly	GGC(50	5	45	27					
					Thr	ACG(26)	5	0	12
Glu	GAG(81)	2	27	27	Thr	ACA(11)	12	0	0
Glu	GAA(19)	25	0	2	Thr	ACT(16)	12	0	4
					Thr	ACC(47)	6	35	20
Asp	GAT(24)	20	0	3					
Asp	GAC(76	5	25	22	Trp	TGG(100)	10	10	10
Val	GTG(40	6	42	32	End	TGA(26)	0	0	0
Val	GTA(6)	18	0	0	End	TAG(52)	0	0	1
Val	GTT(17)	18	0	4	End	TAA(22)	0	0	0
Val	GTC(37)	0	0	6					
					Cys	TGT(21)	1	0	0
Ala	GCG(24)	1	0	0	Cys	TGC(79)	1	2	2
Ala	GCA(13)	11	0	0					
Ala	GCT(27)	19	0	26	Tyr	TAT(14)	23	0	0
Ala	GCC(36)	4	35	10	Tyr	TAC(86)	4	27	27
Arg	AGG(26)	7	0	15	Leu	TTG(13)	5	0	13
Arg	AGA(7)	23	0	0	Leu	TTA(3)	22	0	0
Arg	CGG(13)	1	0	0	Leu	CTG(31)	2	48	20
Arg	CGA(3)	4	0	0	Leu	CTA(9)	8	0	0
Arg	CGT(11)	7	0	4	Leu	CTT(16)	10	0	14
Arg	CGC(40)	1	43	24	Leu	CTC(28)	1	0	2
Ser	AGT(5)	21	0	0	Phe	TTT(20)	30	0	0
Ser	AGC(28)	3	61	23	Phe	TTC(80)	6	36	36
Ser	TCG(16)	7	0	6					
Ser	TCA(10)	12	0	0	Gln	CAG(59)	5	27	8
Ser	TCT(14)	11	0	21	Gln	CAA(41)	22	0	19
Ser	TCC(27)	7	0	11					
					His	CAT(29)	7	0	0
Lys	AAG(90)	1	2	2	His	CAC(71)	2	9	9
Lys	AAA(10)	1	0	0					
					Pro	CCG(27)	6	0	0
Asn	AAT(19)	34	0	0	Pro	CCA(23)	15	0	26
Asn	AAC(81)	14	48	48	Pro	CCT(20)	10	0	9
					Pro	CCC(30)	2	33	0
					Met	ATG(100)	8	8	8

[a] Numbers in parentheses refer to codon usage (%) in maize genes, as explained for Table 2.

Analysis of Mze HD73 #6 trnc+ and comparison to dicots and maize genes is set forth in Table 13.

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Table 13. Deviation of codon usage between MZE HD73 #6 trnc+, dicots and maize.

Amino Acid	Codon	Freq. of Codon Usage in Mze #6[a]	Freq. of Codon Usage in Dicots[b]	Dev. of Codon Usage from Dicots[c]	Freq. of Codon Usage in Maize[b]	Deviation of Codon Usage from Maize[c]
		(Y_n)	(X_n)	A	(X_n)	A
Gly	GGG	0	0.12	1.5625	0.16	1.5625
	GGA	0	0.38	1.5625	0.13	1.5625
	GGT	0.4	0.33	0.3314394	0.21	1.4136905
	GGC	0.6	0.16	4.296875	0.5	0.3125
Glu	GAG	0.93104	0.51	1.2899341	0.81	0.233477
	GAA	0.06896	0.49	1.3425844	0.19	0.9953494
Asp	GAT	0.12	0.58	1.2392241	0.24	0.78125
	GAC	0.88	0.42	1.7113095	0.76	0.2467105
Val	GTG	0.76191	0.29	2.5425903	0.4	1.4136905
	GTA	0	0.12	1.5625	0.06	1.5625
	GTT	0.09524	0.39	1.1809371	0.17	0.6871499
	GTC	0.14286	0.2	0.4464286	0.37	0.9592181
Ala	GCG	0	0.06	1.5625	0.24	1.5625
	GCA	0	0.25	1.5625	0.13	1.5625
	GCT	0.72222	0.42	1.1243386	0.27	2.6170267
	GCC	0.27778	0.27	0.0450103	0.36	0.3568673
Arg	AGG	0.34884	0.25	0.6177326	0.26	0.5338775
	AGA	0	0.3	1.5625	0.07	1.5625
	CGG	0	0.04	1.5625	0.13	1.5625
	CGA	0	0.08	1.5625	0.03	1.5625
	CGT	0.09302	0.21	0.8703627	0.11	0.2411469
	CGC	0.55814	0.11	6.3656184	0.4	0.6177326
Ser	AGT	0	0.14	1.5625	0.05	1.5625
	AGC	0.37705	0.18	1.7104964	0.28	0.5415691
	TCG	0.09836	0.06	0.9989754	0.16	0.6019467
	TCA	0	0.19	1.5625	0.1	1.5625
	TCT	0.34426	0.25	0.5891393	0.14	2.2797131

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	TCC	0.18033	0.18	0.0028461	0.27	0.5189359
Lys	AAG	1	0.61	0.9989754	0.9	0.1736111
	AAA	0	0.39	1.5625	0.1	1.5625
Asn	AAT	0	0.45	1.5625	0.19	1.5625
	AAC	1	0.55	1.2784091	0.81	0.3665123
Ile	ATA	0	0.18	1.5625	0.08	1.5625
	ATT	0.27659	0.45	0.6020981	0.24	0.2382535
	ATC	0.72340	0.37	1.4924166	0.68	0.099734
Thr	ACG	0.33333	0.08	4.9479167	0.26	0.4407051
	ACA	0	0.27	1.5625	0.11	1.5625
	ACT	0.11111	0.35	1.0664683	0.16	0.4774306
	ACC	0.55556	0.3	1.3310185	0.47	0.2844267
Trp	TAG	1	1	0	1	0
End	TGA	0	0.33	1.5625	0.26	1.5625
	TAG	1	0.19	6.6611842	0.52	1.4423077
	TAA	0	0.48	1.5625	0.22	1.5625
Cys	TGT	0	0.44	1.5625	0.21	1.5625
	TGC	1	0.56	1.2276786	0.79	0.4153481
Tyr	TAT	0	0.43	1.5625	0.14	1.5625
	TAC	1	0.57	1.1787281	0.86	0.2543605
Leu	TTG	0.26531	0.26	0.0318878	0.13	1.6262755
	TTA	0	0.1	1.5625	0.03	1.5625
	CTG	0.40816	0.09	5.5236678	0.31	0.4947745
	CTA	0	0.08	1.5625	0.09	1.5625
	CTT	0.28571	0.28	0.0318878	0.16	1.2276786
	CTC	0.04082	0.19	1.2268394	0.28	1.3347303
Phe	TTT	0	0.45	1.5625	0.2	1.5625
	TTC	1	0.55	1.2784091	0.8	0.390625
Gln	CAG	0.29630	0.41	0.433322	0.59	0.777817
	CAA	0.70370	0.59	0.3011221	0.41	1.1192977
His	CAT	0	0.54	1.5625	0.29	1.5625
	CAC	1	0.46	1.8342391	0.71	0.6382042
Pro	CCG	0	0.09	1.5625	0.27	1.5625

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	CCA	0.74286	0.42	1.2011054	0.23	3.4840839
	CCT	0.25714	0.32	0.3069196	0.2	0.4464286
	CCC	0	0.17	1.5625	0.3	1.5625
Met	ATG	1	1	0	1	0
	Total			97.160136		68.584457

[a]Calculations based on codon numbers for MZE HD73 #6 trnc+ from Table 12.

[b]Numbers taken from U.S.P.N. 5,380,831 (Table 1)

[c]Calculations based on formula set forth in Definitions section.

Compared to the bacterial sequence, Mze HD73 #6 trnc+ has 538 base changes within the 1845 bp of the ICP coding region (538/1845 x 100 = 29% difference), and 6 additional changes due to the addition of the two Pro codons, for a total of 544 differences in 1851 bp. Comparison with the DNA sequence published by Perlak et al. (PNAS, 88 (1991) 3324) reveals that the present maize optimized *Bt* ICP gene differs at 422 positions out of 1845 (23% difference), and the encoded proteins differ at amino acids 206, 227, 245, 254, 289, and 313 (6 changes out of 615 amino acids, not including the terminal prolines).

Table 14 set forth below further illustrates the teachings of the method of modifying a gene by using preferred and non-preferred maize codons to make a plant optimized nucleotide sequence.

Table 14. Use of Non-preferred Maize Codons in MZE HD73 #6 trnc+.

	PHE					
Maize(%)	TTC(76)	TTT(24)				
MZE#6(36)	36	0				

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	LEU					
Maize(%)	CTG(28)	CTC(26)	CTT(16)	TTG(15)	CTA(10)	TTA(5)
MZE#6(49)	20	2	14	13	0	0
	SER					
Maize(%)	AGC(26)	TCC(24)	TCG(16)	TCT(14)	TCA(13)	AGT(7)
MZE#6(61)	23	11	6	21	0	0
	TYR					
Maize(%)	TAC(80)	TAT(20)				
MZE#6(27)	27	0				
	END					
Maize(%)	TGA(46)	TAG(42)	TAA(12)			
MZE#6(1)	0	1	0			
	CYS					
Maize(%)	TGC(75)	TGT(25)				
MZE#6(2)	2	0				
	TRP					
Maize(%)	TGG(100)					
MZE#6(10)	10					
	PRO					
Maize(%)	CCG(29)	CCA(26)	CCC(25)	CCT(20)		
MZE#6(35)	0	26	0	9		
	HIS					
Maize(%)	CAC(65)	CAT(35)				
MZE#6(9)	9	0				
	GLN					
Maize(%)	CAG(56)	CAA(44)				
MZE#6(27)	8	19				
	ARG					
Maize(%)	CGC(34)	AGG(30)	CGG(14)	CGT(10)	AGA(8)	CGA(4)
MZE#6(43)	24	15	0	4	0	0
	ILE					
Maize(%)	ATC(60)	ATT(27)	ATA(13)			
MZE#6(47)	34	13	0			
	MET					
Maize(%)	ATG(100)					
MZE#6(8)	8					
	THR					

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Maize(%)	ACC(45)	ACG(22)	ACT(18)	ACA(15)		
MZE#6(36)	20	12	4	0		
	ASN					
Maize(%)	AAC(79)	AAT(21)				
MZE#6(48)	48	0				
	LYS					
Maize(%)	AAG(81)	AAA(19)				
MZE#6(2)	2	0				
	VAL					
Maize(%)	GTG(42)	GTC(33)	GTT(18)	GTA(7)		
MZE#6(42)	32	6	4	0		
	ALA					
Maize(%)	GCC(33)	GCG(26)	GCT(26)	GCA(15)		
MZE#6(36)	10	26	0	0		
	ASP					
Maize(%)	GAC(68)	GAT(32)				
MZE#6(25)	22	3				
	GLU					
Maize(%)	GAG(76)	GAA(24)				
MZE#6(29)	27	2				
	GLY					
Maize(%)	GGC(45)	GGG(21)	GGT(21)	GGA(13)		
MZE#6(45)	27	0	18	0		

In MZE HD73 #6 trnc+, maize codon preferences are distributed as follows:

19 of the 20 first choice codons are used a total of 389 out of 618 possible times, or 63% of the time.

13 of the 18 second choice codons are used a total of 136 times out of 618 possible times, or 22% of the time.

5 of the 10 third choice codons are used a total of 46 times out of 618 possible times, or 7.5% of the time.

6 of the 8 fourth choice codons are used a total of 47 times out of 618 possible times, or 7.5% of the time.

0 of the 3 fifth choice codons are used.

0 of the 3 sixth choice codons are used.

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Based on the frequency of use of first choice maize codons, MZE HD73 #6 trnc+ is 63% homologous to a pure plant optimized nucleotide sequence.

Synthesis of a Maize Optimized *Bt* ICP Gene

A nucleotide sequence corresponding to Mze HD73 #6 trnc+ was synthesized in a series of Polymerase Chain Reactions (PCR) as taught in U.S. Patent No. 4,683,202 to Mullis and U.S. Patent No. 4,683,195 to Mullis et al., by stepwise addition of overlapping oligonucleotides. The procedure relies on PCR amplification of intermediate synthesis product, followed by amplification of extensively modified large DNA fragments prior to cloning. Following one round of amplification, the intermediate product is purified, annealed to the next set of overlapping primers, and amplified. Entire genes can thus be synthesized without annealing, ligation, transformation and selection of intermediate reaction products; steps that are necessary with other approaches.

Taq polymerase, the enzyme used in PCR amplification, lacks 3'-5' exonuclease activity, and therefore is unable to 'proofread' the nascent sequence and remove misincorporated nucleotides. Under certain conditions (55°C annealing temperature and 200 µM deoxynucleotide concentration), the polymerase was calculated to misincorporate nucleotides at a frequency of 5×10^{-6} (Gelfland et al., PCR Protocols, (1989), Academic Press, Inc., San Diego, CA). The chance that an error occurs in a certain sequence increases with increasing numbers of amplification cycles, and therefore larger genes are best synthesized in several separate parts of intermediate size (500-700 nucleotides) which are subsequently sown together by PCR amplification, or joined together by traditional ligation of ends. This strategy also allows the different parts to be modified

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or exchanged without affecting the entire sequence of the gene.

In one aspect of the invention for the design of the *Bt* ICP gene, several unique restriction enzyme recognition sites were introduced into the sequence to allow the joining of separately synthesized parts (SEQ. ID. NO. 1). Also, two C residues were added to the 5' end of the sequence shown in SEQ. ID. NO. 1 to generate an *Nco I* site, and a *BamH I* site was added to the 3' end of the gene (downstream of the coding region) so that the completed ICP gene could be inserted into *Nco I* and *BamH I* (or other sites with *BamH I* compatible ends) sites in some of the vectors. The 1854 nt ICP sequence was split into three parts of roughly equivalent size. Each part, upon completion of the synthesis, was designed to contain at each of its ends a unique restriction site. These sites were used to join the individual parts together to construct the contiguous sequence encoding 617 amino acids. The 5' part was designed to have unique 5' *Nco I* and 3' *Xho I* sites at the ends, the central part was designed to have unique 5' *Xho I* and 3' *Kpn I* sites at the ends, and the 3' part was designed to have unique 5' *Kpn I* and 3' *BamH I* sites (see Figure 1A).

In another aspect of the invention, the 5'-most ICP gene fragment of 653 basepairs (bp) was synthesized from 12 overlapping 61 to 86 base long oligonucleotides in 6 PCR steps. All oligonucleotides were designed to produce 18 to 20 base overlaps during the successive PCR steps. In each case, synthesis of the fragment was carried out from the "inside-out", as is exemplified in Figure 1B. Step 1 of the synthesis process was begun by annealing of oligonucleotides Bt1 and Bt2. Only in the central area of overlap between the two is the annealed molecule double stranded. The remainder of the molecule was made double stranded by extension with the Taq Polymerase during 30 amplification cycles. In the second

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step, this double stranded molecule was denatured, then annealed to and reamplified with oligonucleotides Bt3 and Bt4. In the third step, this double stranded molecule (corresponding to the sequence of Bt3, Bt1, Bt2 and Bt4) was denatured, annealed to and amplified with oligonucleotides Bt5 and Bt6. This process was repeated until the sequence was extended to a 653 bp double stranded molecule corresponding to the entire sequence of the 5' part of the Bt gene (see Figure 1B). Similarly, the 584 bp central fragment was synthesized using 10 overlapping 75 to 83 base long oligonucleotides in 5 PCR steps and the 3'-most ICP gene fragment of 657 bp was synthesized using 12 overlapping 59 to 84 base oligonucleotides in 6 PCR steps. Following synthesis, each of the gene parts cloned in pBlueScript ("pBS", Stratagene, La Jolla, CA) vectors were verified by sequence analysis. Corrections, when necessary, were made using a PCR mutagenesis approach. The corrections were resequenced prior to joining of the individual fragments into the complete gene.

The *Bt* ICP gene was constructed from a total of 34 oligonucleotides ranging in size from 59 to 86 nt. The sequence of all 34 oligonucleotides is presented in Table 15.

Table 15. Oligonucleotides Used in the Synthesis of the *Bt* ICP gene.[a]

Name (SEQ. ID.NO)	Fragment	Sequence	Location	Size
Bt 1 (9)	5' end	CGA GCA GCT CAT CAA CCA ACG CAT TGA GGA GTT CGC TCG CAA CCA AGC CAT CTC CAG GCT TGA GGG CTT GAG CAA CCT GTA	240-330	81
Bt 2 (10)	5' end	CAA GGC TAG GTT GGT TAG GTC AGC CTC CCA CTC CCT GAA GCT CTC AGC GTA GAT TTG GTA CAG GTT GCT CAA GCC CTC	rc 301-378	78
Bt 3 (11)	5' end	CTT CGG CCC AAG CCA ATG GGA CGC TTT CCT TGT GCA AAT CGA GCA GCT CAT CAA CCA ACG CAT TGA GG	201-268	68

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Bt 4 (12)	5' end	GGC CGT GGT CAG AGC AGA GTT CAT GTC GTT GAA TTG GAT GCG CAT CTC CTC CCT CAA GGC TAG GTT GGT TAG G	rc 360-432	73
Bt 5 (13)	5' end	CTG AGT TCG TGC CAG GTG CTG GCT TCG TGC TTG GTC TAG TCG ACA TCA TCT GGG GCA TCT TCG GCC CAA GCC AAT GGG	143-220	78
Bt 6 (14)	5' end	TGC ACG TAG ACG CTC AGA AGA GGC ACT TAG TAG TTC TGC ACA GCG AAC AGA GGG ATG GCC GTG GTC AGA GCA GAG TTC A	rc 410-488	79
Bt 7 (15)	5' end	ACA CCC CAA TCG ACA TCT CTC TGA GCC TGA CCC AAT TCC TGC TGT CTG AGT TCG TGC CAG GTG CTG GC	98-165	71
Bt 8 (16)	5' end	CGA AGC CCC AGC GTT GAC CGA ACA CAG ACA CGT CCC TCA ACA CAG ACA GGT GCA AGT TAG CAG CTT GCA CGT AGA CGC TCA GAA GAG	rc 467-553	86
Bt 9 (17)	5' end	GAG GTG GAG GTG CTT GGT GGT CAG CGC ATT GAG ACC GGC TAC ACC CCA ATC GAC ATC TCT C	58-118	61
Bt 10 (18)	5' end	CAG CCT GGT CAG GTC GTT GTA GCG AGA GTT GAT GGT GGC AGC ATC GAA GCC CCA GCG TTG ACC G	rc 534-597	64
Bt 11 (19)	5' end	CCA TAG ACA ACC CAA ACA TCA ACG AGT GCA TCC CTT ACA ACT GCT TGA GCA ACC CTG AGG TAG AGG TGC TTG GTG G	2-77	79
Bt 12 (20)	5' end	CCT CTC GAG GCC CGT GTT GTA CCA GCG CAC AGC GTA GTC CGT GTA GTT GCC AAT CAG CCT GGT CAG GTC GTT GTA GCG	rc 574-651	78
Bt 13 (21)	center	CGC TCT CCT CAC CTG ATG GAC ATT CTT AAC AGC ATC ACT ATC TAC ACG GAC GCT CAC CGC GGC TAC TAC TAC TAG	874-948	75
Bt 14 (22)	center	GAA CTC TAG GCC AGA GAA ACC AAC TAG GGA AGC CAT GAT TTG GTG GCC AGA CCA GTA GTA GTA GCC GCG GTG AGC	rc 799-925	75
Bt 15 (23)	center	TTG GAG AAC TTC GAT GGC TCC TTC AGG GGC TCT GCT CAA GGC ATT GAG CGC AGC ATT CGC TCT CCT CAC CTG ATG GAC	817-894	78
Bt 16 (24)	center	GTT GAG CCA CAA TGC GCT GTT GTG GAG CAG CGT TGC CCA TCG TAC CGT ACA AAG GGA AGG TGA ACT CTG GGC CAG AGA AAC C	rc 979-1060	82
Bt 17 (25)	center	CCC GTC GCT ACC CAA TCC GCA CGG TGT CTC AGC TGA CTA GGG AGA TTT ACA CCA ACC CAG TTT TAG AGA ACT TCG ATG GCT CC	755-837	83
Bt 18 (26)	center	GTT GAA TAG GCG GCG GTA CAA GGT GCT GGA AAG GGT GCG GTA CAC ACC TTG GCC CAG TTG AGC CAC AAT GCG CTG TTG	rc 1039-1116	78
Bt 19 (27)	center	CCG CAG GGA GCT GAC CCT GAC GGT GCT TGA CAT TGT GGC TCT GTT CCC AAA CTA CGA CTC CCG TCG CTA CCC AAT CCG C	696-774	79
Bt 20 (28)	center	AAG CGA ACT CAG TGC CAT CAA GCA CAG AAA GCT GTT GGT TGT TGA TGC CAA TGT TGA ATG GGC GGC GGT ACA AGG	rc 1094-1168	75
Bt 21 (29)	center	GGC CTC GAG AGG GTG TAG GGT CCA GAC TCC AGG GAC TAG GTG CGT TAC AAC CAA TTC CGC AGG GAG CTG ACC CTG	640-714	75

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Bt 22 (30)	center	GTC CAC GGT ACC AGA CTT GCG GTA CAC AGC GCT TAG CAA GTT GCT AGA GGT GCC GTA AGC GAA CTC AGT GCC ATC AAG	rc 1147-1224	78
Bt 23 (31)	3' end	GTC ATC TCG GGT CCA GGT TTC ACG GGT GGT GAC CTT GTG CGC TTG AAC AGC TCG GGT AAC AAC ATC CAG AAC AGG GGT TAC	1459-1539	81
Bt 24 (32)	3' end	ACC TGA CCC TGA CCC TGT AGC GGG TGC TAG TAG ATG GGA AGT GGA TTG GCA CCT CGA TGT AAC CCC TGT TCT GGA TGT T	rc 1519-1597	79
Bt 25 (33)	3' end	GCC TCT GAC AGC ATC ACT CAA ATC CCT GCT GTT AAG GGC AAC TTC CTT TTC AAC GGC TCG GTC ATC TCG GGT CCA GGT TTC	1399-1470	72
Bt 26 (34)	3' end	GTT GCT GAA GAT GGA GCT GTT GCC CCA GTT CAC GTT CAA GTG GAT TAG GGT GGT CAC AGA AGC GTA CCT GAC CCT GAC CCT GTA	rc 1579-1659	81
Bt 27 (35)	3' end	ATC AGG GCC CCA ATG TTC TCC TAG ATT CAC CGT TCT GCT GAG TTC AAC AAC ATC ATT GCC TCT GAC AGC ATC ACT CAA	1342-1419	78
Bt 28 (36)	3' end	CGA AGT AGC CGA AGT CCG AGG ACT GCA GGT TGT CAA GGC TAG TAG CCG TAG CTG GCA CCG TGT TGC TGA AGA TAG AGC TGT T	rc 1639-1720	82
Bt 29 (37)	3' end	CAC CGC TTG AGC CAC GTT TCC ATG TTC CGT TCG GGC TTC AGC AAC AGC TCT GTG AGC ATT ATC AGG GCC CCA ATG TTC	1282-1359	78
Bt 30 (38)	3' end	GCG CAC ACC CAC GAT GTT GCC AAG AGA GCT GGT GAA AGC GTT GGC CGA TTC GAA GTA GCC GAA GTC CGA	rc 1702-1770	69
Bt 31 (39)	3' end	CCA CAG AAC AAC AAC GTG CCA CCT CGC CAA GGC TTC TCT CAC CGC TTG AGC CAC GTT TCC	1243-1302	60
Bt 32 (40)	3' end	GGG ATG AAT TCG AAG CGG TCG ATG ATG ACA CCA GCC GTG CCA GAG AAG TTG CGC ACA CCC ACG ATG TTG CC	rc 1750-1820	71
Bt 33 (41)	3' end	CGC AAG TCT GGT ACC GTG GAC AGC TTG GAC GAG ATC CCT CCA CAG AAC AAC AAC GTG CCA	1204-1263	60
Bt 34 (42)	3' end	CTC TAG ATC CCT ATG GTG GCT CAG CCT CCA GGG TAG CCG TCA CTG GGA TGA ATT CGA AGC GGT C	rc 1801-1854	64

[a] For each oligonucleotide, the name, gene fragment sequence, location in the complete ICP gene, and length (in bases) are shown. Nucleotide locations marked with rc indicate that the sequence of the oligonucleotide corresponds to the reverse complement of the nucleotide sequence of the top (coding) strand of the gene.

Several conditions were followed in the design of the oligonucleotides: i) All oligonucleotide overlaps were a minimum of 18 nt; ii) The 3'-most base of each oligonucleotide was chosen to be G or C; iii) The 5'-most base of each oligonucleotide was chosen adjacent to and downstream of a T residue in the sequence, in order

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to avoid problems with non-template addition of A residues at the 3' end of the opposite strand (Clark et al., Nucl. Acids Res., 16 (1988) 9677); iv) Extensive internal basepairing in each oligonucleotide was avoided where possible; and v) Basepairing between oligonucleotides used in all steps except the first (oligonucleotide annealing) step for each fragment was also avoided where possible.

Gene Expression in *E. coli*

To demonstrate that a functional protein of correct size, antigenicity, and toxicity to Lepidopteran insects was encoded by the synthesized nucleotide sequence, expression studies were carried out in *E. coli* prior to the initiation of plant transformation experiments. To this end, the maize optimized DNA sequence coding for ICP was inserted in T7 expression plasmids, and *E. coli* extracts highly enriched in the ICP gene product were prepared. SDS-PAGE and immunoblot analysis demonstrated that the gene product was of the correct size and cross reacted with antiserum raised against purified native *B. thuringiensis* delta-endotoxin (Figure 4). The biological action of the protein was demonstrated in *M. sexta* feeding assays (Figure 5). Further verification of the success of engineering and synthesis strategies was provided by the demonstration that the ICP gene produced antigenically active protein of the correct size in transformed maize callus cells (Figure 6). Feeding bioassays with *H. virescens* larvae revealed the insecticidal activity of the engineered protein. Together, these data demonstrate that the maize optimized nucleotide sequence produces a protein which shares several biological features (e.g., antigenicity, size, biological activity) with wildtype ICP isolated from nature.

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Preparation of Recombinant DNA Vectors Containing the Synthetic Maize Optimized *Bt* ICP Gene

The maize optimized nucleotide sequence coding for *Bt* ICP is expressed in plants at an enhanced level when compared to that observed with natural *Bt* structural genes. Expression of the maize optimized *Bt* ICP nucleotide sequence requires transformation of a plant cell with an appropriate vector. The maize optimized nucleotide sequence for *Bt* ICP was combined with a promoter functional in plants, where the structural gene and the promoter region being in such position and orientation with respect to each other that the structural gene can be expressed in a cell in which the promoter region is active, thereby forming a functional gene. The promoter regions include, but are not limited to, bacterial and plant promoter regions. In another aspect of the invention, the promoter is selected from the group consisting of inducible promoters, constitutive promoters, temporal or developmentally-regulated promoters, tissue-preferred, and tissue-specific promoters.

In an important aspect of the invention, the vector includes an MSV (Maize Streak Virus) leader sequence, a 35S promoter, and an enhancer specific for maize, such as an Adh intron 1 or Adh intron 6 as further described in the Examples.

To express the promoter region/structural gene combination, the DNA segment carrying the combination is contained by a cell. Combinations which include plant promoter regions are contained by plant cells, which, in turn, may be contained by plants or seeds. Combinations which include bacterial promoter regions are contained by bacteria, e.g., *Bt* or *E. coli*. Those in the art will recognize that expression in types of micro-organisms other than bacteria may in some circumstances be

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desirable and, given the present disclosure, feasible without undue experimentation.

Appropriate recombinant DNA vectors with which the maize optimized *Bt* ICP gene can be combined are further described in herein in the Examples.

Transformation of Maize with the Synthetic ICP Gene Vector and Transformation of all Plants with the Doubly Enhanced Promoter

The recombinant DNA molecule carrying a maize optimized *Bt* ICP gene under promoter control can be introduced into plant tissue by any means known to those skilled in the art. The technique used for a given plant species or specific type of plant tissue depends on the known successful techniques. As novel means are developed for the stable insertion of foreign genes into plant cells and for manipulating the modified cells, skilled artisans will be able to select from known means to achieve a desired result.

The doubly enhanced promoters can be used to express foreign genes in maize as well as dicots or other monocots. More specifically, dicots include but are not limited to soybeans, legumes, rapeseed, cotton, sunflower, tomatoes, potatoes, sugar beets, alfalfa, cloves and peanuts. Monocots include but are not limited to maize, wheat, sorghum, oats, rye, barley, rice, millets, sugar cane and grasses.

In addition to using a doubly enhanced 35S or 19S promoter from cauliflower mosaic virus, other promoters may be modified by the teachings discussed herein. More specifically, promoters which may be modified with the MSV leader sequence adh1, adh6, or other introns (SEQ. ID. NOS. 43, 44, 45, 46 and 47) include but are not limited to octopine synthase

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promoter, nopaline synthase promoter and manopine synthetase promoter.

Plant promoters, can also be further modified by the teachings herein and include but are not limited to ribulose-1,6-biphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, phaseolin promoter, ADH promoter, actin, ubiquitin, zein, oleosin, napin, ACP, heat-shock promoters, and tissue specific promoters or pollen-specific, embryo specific, corn silk specific, cotton fiber specific, root specific, seed endosperm specific promoters and the like.

Several techniques exist for introducing foreign genetic material into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include acceleration of genetic material coated onto microparticles directly into cells (US Patents 4,945,050 to Cornell and 5,141,131 to DowElanco). Plants may be transformed using Agrobacterium technology, see US Patent 5,177,010 to University of Toledo, 5,104,310 to Texas A&M, European Patent Application 0131624B1, European Patent Applications 120516, 159418B1, European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot, US Patents 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot, European Patent Applications 116718, 290799, 320500 all to MaxPlanck, European Patent Applications 604662 and 627752 to Japan Tobacco, European Patent Applications 0267159, and 0292435 and US Patent 5,231,019 all to Ciba Geigy, US Patents 5,463,174 and 4,762,785 both to Calgene, and US Patents 5,004,863 and 5,159,135 both to Agracetus. Other transformation technology includes whiskers technology, see Us Patents 5,302,523 and 5,464,765 both to Zeneca. Electroporation technology has also been used to transform plants, see WO 87/06614 to Boyce Thompson Institute, 5,472,869 and 5,384,253 both to Dekalb,

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WO9209696 and WO9321335 both to PGS. All of these transformation patents and publications are incorporated by reference. In addition to numerous technologies for transforming plants, the type of tissue which is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue type I and II, hypocotyl, meristem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques within the skill of an artisan.

Another variable is the choice of a selectable marker. The preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which code for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bar); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorosulfuron; bromoxynil, dalapon and the like.

In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used without a selectable marker. Reporter genes are genes which are typically not present or expressed in the recipient organism or tissue. The reporter gene typically encodes for a protein which provide for some phenotypic change or enzymatic property. Examples of such genes are provided in K. Weising et al. Ann. Rev. Genetics, 22, 421 (1988), which is incorporated herein by reference. A preferred reporter gene is the glucuronidase (GUS) gene.

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Once introduced into the plant tissue, the expression of the structural gene may be assayed by any means known to the art, and expression may be measured as mRNA transcribed or as protein synthesized. Techniques are known for the in vitro culture of plant tissue, and in a number of cases, for regeneration into whole plants (EP Appln No. 88810309.0). Procedures for transferring the introduced expression complex to commercially useful cultivars are known to those skilled in the art.

Once plant cells expressing the gene under control of a plant expressible promoter are obtained, plant tissues and whole plants can be regenerated therefrom using methods and techniques well-known in the art. The regenerated plants are then reproduced by conventional means and the introduced genes can be transferred to other strains and cultivars by conventional plant breeding techniques.

Expression of the ICP Gene in Maize Cells

The functionality of the maize optimized *Bt* ICP gene in plant cells has been tested in maize transformation systems, in Black Mexican Sweet (BMS) protoplasts, and in stably transformed maize callus cultures. These studies indicated that the engineered ICP gene expressed well in maize and that the levels of accumulated ICP were sufficient to provide insect control in *in vitro* feeding assays.

Introduction of the gene into regenerable maize cultures by helium blast transformation as described in U.S. Patent No. 5,141,131, provided fertile plants that expressed the gene. Plants grown from the seeds of transgenic maize plants also expressed the ICP gene in subsequent generations.

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The following examples illustrate methods for carrying out the invention and should be understood to be illustrative of, but not limiting upon, the scope of the invention which is defined in the appended claims.

EXAMPLES

EXAMPLE 1: Oligonucleotide Synthesis.

Oligonucleotides were synthesized on either the Applied Biosystems Inc., DNA synthesizer model 380A or model 390 using 0.2 µM columns and FOD phosphoramidites and standard cyanoethyl chemistry; synthesis was done in the Trityl-Off mode. Following synthesis on the Model 380A synthesizer, each oligonucleotide was cleaved off the column and deprotected at 50°C for 1 hr. and dried down by evaporation at 50°C. The oligonucleotides were resuspended in 300 µl TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA) and the concentration was determined by measuring the absorbance at 260 nm.

Oligonucleotides were purified by electrophoresis on 12% denaturing polyacrylamide gels (PAGE). A PAGE gel stock solution of 300 mls was made by dissolving 126 g urea in 30 ml 10x Tris Borate EDTA buffer (TBE; 1x TBE is 0.9 M Tris-borate, 2 mM EDTA) and 90 ml 40% acrylamide stock and adjusting the volume of the solution to 300 mls with H_2O. The gel solution was filtered through a 0.2 µm filter. 40 mls of the PAGE stock was used to pour a 5-well gel using a Hoeffer Sturdier gel apparatus. Polymerization was initiated by addition of 350 µl 10% ammonium persulfate and 35 µl TEMED prior to pouring.

Each oligonucleotide was prepared as follows: 300 to 500 µg of oligonucleotide was diluted to 60 µl

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with TE buffer, then 60 µl of formamide gel loading buffer (10 ml formamide, 10 mg xylene cyanol FF, 10 mg bromophenol blue, 200 µl 0.5 M EDTA pH 8.0) was added and the sample was boiled for 5 minutes and chilled on ice. The samples were loaded on the gel using a sequencing pipet tip. Electrophoresis was carried out in 1x TBE at 300 volts for 3 hrs.

Following the run the acrylamide gel was transferred to SaranWrap™, placed on a white background (e.g., X-Ray intensifying screen), and exposed to short wave UV light. The presence of the DNA bands, as well as the location of xylene cyanol and bromophenol blue dye markers, was visualized as a shadow on the white background.

The DNA bands of appropriate size were excised from the gel and the DNA was eluted by diffusion. Each gel slice was macerated with a glass rod and incubated in 1.5 ml of oligo elution buffer (100 mM Tris HCl pH 8.0, 500 mM NaCl, 5 mM EDTA) with constant agitation in a rolling drum at 37°C for 16 hours. The polyacrylamide slurry was filtered through a 3 cc syringe containing a glasswool plug and an attached 0.2 µm filter. The eluted oligonucleotide was concentrated by centrifugation for 2 hrs. at 3000 x g in a Centricon 10 spin column (molecular weight cut-off 10,000 D) at room temperature, and washed with 2 ml TE buffer by centrifugation as above in the same tube. The purified oligonucleotide was recovered in a final volume of 30 to 40 µl. Concentration was determined by measurement of the absorbance at 260 nm.

As an example of the result of oligonucleotide synthesis, the gel purification of oligonucleotides Bt6 - Bt10 is shown in Figure 2. Figure 2 also shows two successful syntheses with the 380A synthesizer (Bt9 and Bt10) and two successful syntheses with the 390 synthesizer (Bt6 and Bt7).

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EXAMPLE 2: PCR Amplification.

All PCR amplifications were done in 100 µl reactions containing 20 mM Tris HCl pH 8.3, 1.5 mM $MgCl_2$, 25 mM KCl, 200 µM each of dATP, dGTP, dCTP and dTTP, and 5 units of Taq Polymerase (Perkin Elmer Cetus). Template and PCR primer concentrations varied depending on the step in the protocol. In the first PCR step, template was generated for each fragment by amplification with 0.5 µM of each of the primers of the first set (see Figure 1) in the following regime: 1 minute denaturation at 94°C, 2 minutes annealing at 55°C and 3 minutes extension at 72°C for 30 cycles, followed by an additional extension period of 7 minutes at 72°C. The reaction products were loaded on a 5% native polyacrylamide gel and electrophoresed at 40 volts for 2.5 hours in 1x TBE. BRL 123 bp ladder run in a parallel lane was used as size standard. Following electrophoresis the gel was stained for 1 hr. in water containing 0.5 µg/ml ethidium bromide. The fragments of expected size were cut out of the gel and purified from the gel slice as described for the oligonucleotide purification (see above), with the exception that following filtering through glasswool and 0.2 µm filter, the DNA was concentrated by precipitation with 2.5 volumes of ethanol, 20 µg glycogen, and 0.05 volume of 8 M LiCl. The DNA was resuspended in 40 µl TE buffer. The second PCR step in the synthesis of each fragment was carried out in the same reaction mixture as the first step except that 5 µl of gel purified product of step 1 was used as template and the oligonucleotide concentration was 0.2 µM. The entire PCR reaction was electrophoresed on a 1% agarose gel and the bands of the expected size were excised and DNA was purified from the gel slices using the GeneClean Kit (Bi0101) and eluted in a final volume of 50 µl TE. All subsequent reactions were done as described for step 2.

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Each of the individual PCR steps gave large amounts of product of the expected size. In addition, in most cases bands of double the expected size could be seen, as well as other minor bands. All DNA products of appropriate size were gel purified and run on the gel shown in Figure 3. This figure clearly demonstrates the stepwise addition of DNA sequence in consecutive PCR steps. The dimer-sized bands in each lane are considered to be artifacts of the electrophoresis, because gel purified DNA from monomer-size bands when re-run on a gel also gave this dimer-size band. The final product for each of the gene fragments was digested with the enzymes recognizing the restriction sites built in the end of each fragment (see Figure 1A) and ligated to pBS DNA cut with the same enzymes. The ligation products were transformed into competent *E. coli* DH5α cells, and isolates carrying pBS plasmids containing the appropriate fragments were identified. DNA sequence of the ICP gene portion of these plasmids was determined, and five nucleotide differences from the Mze HD73 #6 trnc+ sequence were found. These changes were: 1) a conservative base change (G to T) in the 5' fragment at nt 639. (The "A" of the ATG start codon is designated base #1); 2) a conservative base change (A to G) in the center fragment at nt 1038; 3) a deletion of two G nucleotides in the center fragment at nt 657-658 which would cause a frameshift in the encoded polypeptide; 4) a base change (T to C) in the center fragment at nt 877 which would result in a serine to proline change; and 5) a deletion of one C nucleotide in the 3' fragment at nt 1401, also producing a frameshift. The latter three errors, which would have resulted in extensive frameshift and amino acid changes, were corrected by PCR mutagenesis as described in Example 3 (see below). Following the PCR correction, the center and 3' fragments were digested and cloned in pBS and the inserts of the resulting plasmids were sequenced to verify that during the correction process no other changes were introduced. Aside from the

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already existing conservative base changes in the 5' and center fragments (which were not corrected) the sequences were identical to the designed ICP (Mze HD73 #6 trnc+) sequence of SEQ. ID. NO. 1.

EXAMPLE 3: Correction of ICP Gene Fragments.

All DNA manipulations and *E. coli* transformations were carried out according to standard procedures (Sambrook et al., Molecular Cloning: A Laboratory Manual, (1989) 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY; Ausubel et al., Current Protocols in Molecular Biology, (1987) John Wiley and Sons, New York, NY). Following cloning of the three individual ICP gene fragments into pBluescript, the sequence was determined using the Sequenase Kit (US Biochemical, Cleveland, OH) using sequencing primers based on the modified ICP sequence or using some of the PCR synthesis primers described above.

Errors in the ICP gene fragments were corrected by PCR mutagenesis. For each correction two PCR reactions were set up. One PCR reaction amplified the 5' half of the fragment, using a 5' end oligonucleotide and the error-correcting oligonucleotide. The other PCR reaction amplified the 3' half of the fragment using the complementary error correction oligonucleotide and a 3' end oligonucleotide. The 5' and 3' corrected fragments were gel purified and joined together in a second step PCR reaction by amplification with only the 5' end and 3' end oligonucleotides as primers. Oligonucleotides used in the error correction were synthesized and gel purified as described above. PCR reaction conditions were as described above, except that annealing was done at 50°C

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and 25 cycles were used. Fragments were gel purified using the GeneClean kit available from Bi0101.

EXAMPLE 4: E. coli Expression.

For *E. coli* expression, the ICP was inserted as a 1862 base pair *Nco I BamH I* DNA fragment in the *Nco I* and *BamH I* sites of cytoplasmic expression vector pET-9d (Novagen, Madison, WI.). One microgram of plasmid was transformed into 0.2 mls of competent cells of *E. coli* strain BL21 (which was available for purchase from Novagen, Madison, WI.) and plated on LB plates containing kanamycin at 25 µg/ml (for plasmid pET-9d). Following overnight incubation at 37°C, colonies were scraped off the plate and resuspended in 10 mls of LB Broth containing the appropriate antibiotic and isopropyl-ß-D-thiogalactoside (IPTG) at 1 mM. Cells were allowed to express protein for 3 hrs. during vigorous shaking at 37°C, then harvested by centrifugation at 1000 x g for 10 minutes at 4°C.

For expression of pET-9d constructs, soluble and aggregated protein fractions were prepared as follows. Cell pellet was frozen and thawed twice, to aid in cell lysis, and the lysate was resuspended in 1 ml of lysis buffer (10mM Tris HCl pH 8.0, 1mM EDTA, 150 mM NaCl, 0.1% Triton X100, 100 g/ml DNase1, 100 ug/ml RNaseH, 1 mg/ml lysozyme) and incubated at 37°C until it was no longer viscous. Soluble protein was separated from aggregated denatured proteins by centrifugation at 4°C for ten minutes. The insoluble pellet was resuspended in approximately 300 ul of the above lysis buffer. Both fractions had a final volume of 0.5 ml.

An abundant protein of molecular size 69 kD was present in the pellet fraction of both extracts produced from *E. coli* cells containing the cytoplasmic expression vector. This protein cross reacted with anti-serum

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raised against native delta-endotoxin purified from *B. thuringiensis* CrylA(c) cultures; the results of a representative protein gel immunoblot are shown in Figure 4. Both *E. coli* (lane 1) and the pellet fraction prepared from the extract of those cells containing cytoplasmic expression vector (lanes 2 and 3) contained cross reactive protein. No cross reactive proteins were seen in equivalent amounts of pellet proteins of extract prepared from cells containing the cytoplasmic expression vector (lane 4).

The size of the anti-ICP cross-reactive protein produced in *E. coli* corresponds closely to the size of 68 kD predicted by the sequence of the ICP gene. The native ICP is slightly smaller (Mw 66 kD) compared to the product of the modified ICP gene (lanes 5, 6 and 7). In *B. thuringiensis*, the toxin is produced as a 130 kD protoxin. Upon ingestion by Lepidopteran insects, the protoxin is solubilized and activated by proteolytic cleavage. This proteolysis produces an active toxin moiety of 60-70 kD, depending on the strain of *B. thuringiensis*. In all Cryl ICPs, proteolytic processing occurs in the center of the protoxin, and separates the toxin moiety from the C-terminal domain. Processing also occurs at the extreme N-terminus between amino acids Arg 28 and Ile 29, and is probably carried out by a serine-type protease. Amino terminal protein sequencing of trypsin activated protoxin of CrylA(b) and CrylC identified isoleucine at position 29 as the N-terminus (Hofte et al., Microbiological Rev., 53 (1989) 242). Since the sequence of the Mze HD73 #6 trnc+ gene includes this putative serine protease site, cleavage at this site by serine protease activity in the *E. coli* extract would remove the N-terminal 28 amino acids. The result would be a product that is ≈3 kD smaller than the gene product predicted from the sequence of the gene. A protein of this size is seen as a faint band (at ≈66 kD) which comigrates with the native ICP toxin. The extracted

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protein was not quantified because the protein itself is insoluble and aggregates with cellular debris.

EXAMPLE 5: Protein Concentrations Expressed by E. coli.

Protein concentrations were determined using the BioRad protein assay. Proteins were analyzed on 12.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) made in a Hoeffer Mighty Small minigel device, or on a Daiichi minigel device according to the manufacturers' recommendations. Staining for protein was done as described (Sambrook et al., Molecular Cloning: A Laboratory Manual, (1989), 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY), ICP was specifically detected by protein gel blot analysis (Western blotting) with rabbit antiserum raised against purified *B. thuringiensis* HD73 toxin, using the ECL Western blotting and detection system (Amersham, Arlington Heights, IL). Proteins were transferred from the gel to a Hybond-ECL nitrocellulose membrane (Amersham) by transfer using a Hoeffer SemiDry blotter at 0.5 mA/cm^2 of gel for 90 min. The membrane was incubated with the blocking reagent TBS-Tween-Milk (TBTM: 25 mM Tris HCl pH 7.4, 136 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, 5% non-fat drymilk) at room temperature for 1 hr. Next, the membrane was incubated with primary antiserum at a 1:500 dilution in blocking reagent, followed by washing three times in 100 ml TBS-Tween (no milk) at room temperature for 10 minutes. The membrane was incubated for 1 hr. in blocking reagent containing secondary antiserum (goat anti-rabbit-IgG conjugated to horseradish peroxidase; Bio-Rad Laboratories, Hercules, CA), then washed three times in 100 ml TBS-Tween at room temperature for 10 minutes. The filter was incubated in 10 mls of reagent A+B (1:1; ECL kit) for 1 minute, excess liquid was drained off, and the membrane was exposed to Hyperfilm-ECL film for 10 sec. to 1 minute. ECL film was processed using standard developer and fixer. ICP signals were scanned with a

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Model 620 video densitometer (Bio-Rad) and the concentration was determined by comparison with scans of ICP standards electrophoresed on the same gel using 1-D Analyst software (Bio-Rad). Figure 4 illustrates expression of ICP in *E. coli* and the concentration of such expressions.

EXAMPLE 6: Feeding Assays.

ICP expressed in *E. coli* and extracted as indicated in Example 4 was used for feeding assays in *Manduca sexta* (tobacco hornworm). Neonate larvae were allowed to feed on artificial diets into which ICP or control samples were incorporated. After 4 days, their weights and mortality were determined.

The results of testing of *E. coli* extracts in *M. sexta* feeding assays (Figure 5) indicated that the ICP encoded by Mze HD73 #6 trnc+ has Lepidopteran toxicity. Both the pellet fraction of *E. coli* extracts, as well as cells expressing the ICP, caused significant growth inhibition and mortality. However, the ICP containing *E. coli* extracts and cells were less toxic to *Manduca* larvae than was the purified native ICP. This may be explained by the fact that the ICP produced in *E. coli* was highly insoluble. It is possible that, due to the aggregation, the effective ICP concentration is much lower than the protein concentration would indicate.

EXAMPLE 7: Construction of Plant Expression Plasmids.

A. Construction of a doubly-enhanced CaMV 35S Promoter: This section describes molecular manipulations which result in a duplication of the expression-enhancer element of a plant promoter. This duplication has been shown (Kay et al., Science 236 (1987) 1299) to result in increased expression in tobacco plants of marker genes

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whose expression is controlled by such a modified promoter. [Note: The sequences referred to in this discussion are derived from the Cabb S strain of Cauliflower Mosaic Virus (CaMV). They are available as the MCASTRAS sequence of GenBank, and published by Franck et al. (Cell 21 (1980) 285). All of the DNA sequences are given in the conventional 5' to 3' direction. The starting material is plasmid **pUC13/35S**(-343) as described by Odell et al. (Nature 313 (1985) 810). This plasmid comprises, starting at the 3' end of the Sma I site of pUC13 (Messing, Methods in Enzymology 101 (1983) 20), and reading on the strand contiguous to the noncoding strand of the lacZ gene of pUC13, nucleotides 6495 to 6972 of CaMV, followed by the linker sequence CATCGATG (which encodes a Cla I recognition site), followed by CaMV nucleotides 7089 to 7443, followed by the linker sequence CAAGCTTG, the latter sequence comprising the recognition sequence for Hind III, which is then followed by the remainder of the pUC13 plasmid DNA.

1. **pUC13/35S**(-343) DNA was digested with Cla I and Nco I, the 3429 base pair (bp) large fragment was separated from the 66 bp small fragment by agarose gel electrophoresis, and then purified by standard methods.

2. **pUC13/35S**(-343) DNA was digested with Cla I, and the protruding ends were made flush by treatment with T4 DNA polymerase. The blunt-ended DNA was the ligated to synthetic oligonucleotide linkers having the sequence CCCATGGG, which includes an Nco I recognition site. The ligation reaction was transformed into competent *Escherichia coli* cells, and a transformant was identified that contained a plasmid (named pOO#1) that had an Nco I site positioned at the former Cla I site. DNA of pOO#1 was digested with Nco I and the compatible ends of the large fragment were religated, resulting in the deletion of 70 bp from **pOO#1**, to generate intermediate plasmid **pOO#1 Nco Δ**.

3. **pOO#1 Nco Δ** DNA was digested with EcoR V, and the blunt ends were ligated to Cla I linkers having the

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sequence CATCGATG. An *E. coli* transformant harboring a plasmid having a new Cla I site at the position of the previous EcoR V site was identified, and the plasmid was named **pOO#1 Nco Δ RV>Cla**.

4. DNA of **pOO#1 Nco Δ RV>Cla** DNA was digested with Cla I and Nco I, and the small (268 bp) fragment was purifed from an agarose gel. This fragment was then ligated to the 3429 bp Cla I/Nco I fragment of pUC13/35S(-343) prepared above in step 1, and an *E. coli* transformant that harbored a plasmid having Cla I/Nco I fragments 3429 and 268 bp was identified. This plasmid was named **pUC13/35S En**.

5. **pUC13/35S En** DNA was digested with Nco I, and the protruding ends were made blunt by treatment with T4 DNA polymerase. The treated DNA was then cut with Sma I, and was ligated to Bgl II linkers having the sequence CAGATCTG. An *E. coli* transformant that harbored a plasmid in which the 416 bp Sma I/Nco I fragment had been replaced with at least two copies of the Bgl II linkers was identified, and named **p35S En²**. [NOTE: The tandomization of these Bgl II linkers generate, besides Bgl II recognition sites, also Pst I recognition sites, CTGCAG].

The DNA structure of **p35s En²** is as follows: Beginning with the nucleotide that follows the third C residue of the Sma I site on the strand contiguous to the noncoding strand of the *lacZ* gene of pUC13; the linker sequence CAGATCTGCAGATCTGCATGGGCGATG (SEQ. ID. NO. 48), followed by CaMV nucleotides 7090 to 7344, followed by the Cla I linker sequence CATCGATG, followed by CaMV nucleotides 7089 to 7443, followed by the Hind III linker sequence CAAGCTT, followed by the rest of pUC13 sequence. This structure has the feature that the enhancer sequences of the CaMV 35S promoter, which lie in the region upstream of the EcoR V site in the viral genome (nts 7090 to 7344), have been duplicated. This promoter construct incorporates the native 35S transcription start

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site, which lies 11 nucleotides upstream of the first A residue of the Hind III site.

Example 7B.

Plasmids utilizing the 35S promoter and the *Agrobacterium* NOS Poly A sequences: The starting material for the first construct is plasmid **pBI221**, purchased from CLONTECH (Palo Alto, CA). This plasmid contains a slightly modified copy of the CaMV 35S promoter, as described in Bevan et al. (1985), Baulcombe et al., (1986), Jefferson et al., (1986, 1987) and Jefferson (1987). Beginning at the 3' end of the Pst I site of pUC19 (Yanisch-Perron et al., 1985), and reading on the same strand as that which encodes the *lacZ* gene of pUC19, the sequence is comprised of the linker nucleotides GTCCCC, followed by CaMV nucleotides 6605 to 7439 (as described in Example 7A), followed by the linker sequence GGGGACTCTAGAGGATCCCCGGGTGGTC AGTCCCTT (SEQ. ID. NO. 49), wherein the underlined bases represent the BamH I recognition sequence. These bases are then followed by 1809 bp comprising the coding sequence of the E. coli uidA gene, which encodes the β-glucuronidase (GUS) protein, and 55 bp of 3' flanking bases that are derived from the E. coli genome (Jefferson, 1986), followed by the Sac I linker sequence GAGCTC, which is then followed by the linker sequence GAATTTCCCC (SEQ. ID. NO. 50). These bases are followed by the RNA transcription termination/polyadenylation signal sequences derived from the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene, and comprise the 256 bp Sau3A I fragment corresponding to nucleotides 1298 to 1554 of DePicker et al. (1982), followed by two C residues, the Eco RI recognition sequence GAATTC, and the rest of pUC19.

1. **pBI221** DNA was digested with EcoR I and BamH I, and the 3507 bp fragment was purified from an agarose gel. **pRAJ275** (CLONTECH, Jefferson, 1987) DNA was digested with EcoR I and Sal I, and the 1862 bp fragment was purified

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from an agarose gel. These two fragments were mixed together, and complementary synthetic oligonucleotides having the sequence GATCCGGATCCG (SEQ. ID. NO. 51) and TCGACGGATCCG (SEQ. ID. NO. 52) were added. [These oligonucleotides when annealed have protruding single-stranded ends compatible with the protruding ends generated by BamH I and Sal I.] The fragments were ligated together, and an *E.coli* transformant harboring a plasmid having the appropriate DNA structure was identified by restriction enzyme analysis. DNA of this plasmid, named **pKA881**, was digested with Bal I and Eco RI, and the 4148 bp fragment was isolated from an agarose gel. DNA pBI221 was similarly digested, and the 1517 bp Eco RI/Bal I fragment was gel purified and ligated to the above pKA881 fragment, to generate plasmid **pKA882**.

2. **pKA882** DNA was digested with Sac I, the protruding ends were made blunt by treatment with T4 DNA polymerase, and the fragment was ligated to synthetic BamH I linkers having the sequence CGGATCCG. An *E.coli* transformant that harbored a plasmid having BamH I fragments of 3784 and 1885 bp was identified and named **pKA882B**.

3. **pKA882B** DNA was digested with BamH I, and the mixture of fragments was ligated. An *E.coli* transformant that harbored a plasmid that generated a single 3783 bp fragment upon digestion with BamH I was identified and named **p35S/NOS**. This plasmic has the essential DNA structure of pBI221, except that the coding sequences of the GUS gene have been deleted. Therefore, CaMV nucleotides 6605 to 7439 are followed by the linker sequence GGGGACTCTAGAGGATCCCGAATTTCCCC (SEQ. ID. NO. 53), where the single underlined bases represent an Xba I site, and the double underlined bases represent a BamH I site. The linker sequence is then followed by the NOS Polyadenylation sequences and the rest of **pBI221**.

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4. **p35S/NOS** DNA was digested with EcoR V and Pst I, and the 3037 bp fragment was purified and ligated to the 534 bp fragment obtained from digestion of p35S En^2 DNA with EcoR V and Pst I. An *E. coli* transformant was identified that harbored a plasmid that generated fragments of 3031 and 534 bp upon digestion with EcoR V and Pst I, and the plasmid was named **p35S En^2/NOS**. This plasmid contains the duplicated 35S promoter enhancer region described for **p35S En^2** in Example 7A Step 5, the promoter sequences being separated from the NOS polyadenylation sequences by linker sequences that include unique Xba I and BamH I sites.

Example 7C

Construction of a synthetic untranslated leader

This example describes the molecular manipulations used to construct a DNA fragment that includes sequences which comprise the 5' untranslated leader portion of the major rightward transcript of the Maize Streak Virus (MSV) genome. The MSV genomic sequence was published by Mullineaux et al., (1984), and Howell (1984), and the transcript was described by Fenoll et al. (1988). The entire sequence, comprising 154 bp, was constructed in three stages (A, B, and C) by assembling blocks of synthetic oligonucleotides.

1. **The A Block:** Complementary oligonucleotides having the sequence
GATCCAGCTGAAGGCTCGACAAGGCAGATCCACGGAGGAGCTGATATTTGGTGGACA (SEQ. ID. NO. 54) and
AGCTTGTCCACCAAATATCAGCTCCTCCGTGGATCTGCCTTGTCCAGCCTTCAGCTG (SEQ. ID. NO. 55) were synthesized and purified by standard procedures. Annealing of these nucleotides into double-stranded structures leaves 4-base single stranded protruding ends [hereinafter referred to as "sticky ends"] that are compatible with those generated by BamH I on one end of the molecule (GATC), and with Hind III-generated single stranded ends on the other end of the

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molecule (AGCT). Such annealed molecules were ligated into plasmid pBluescript SK(-) [hereinafter called pBSK; Stratagene Cloning Systems, LaJolla, CA], that had been digested with BamH I and Hind III. The sequence of these oligonucleotides is such that, when ligated onto the respective BamH I and Hind III sticky ends, the sequences of the respective recognition sites are maintained. An *E. coli* transformant harboring a plasmid containing the oligonucleotide sequence was identified by restriction enzyme analysis, and the plasmid was named **pMSV A**.

2. **The B Block**: Complementary oligonucleotides having the sequences
AGCTGTGGATAGGAGCAACCCTATCCCTAATATACCAGCACCACCAAGTCAGGGCAA
TCCCGGG (SEQ. ID. NO. 56) and
TCGACCCGGGATTGCCCTGACTTGGTGGTGCTGGTATATTAGGGATAGGGTTGCTCC
TATCCAC (SEQ. ID. NO. 57) were synthesized and purified by standard procedures. The underlined bases represent the recognition sequence for restriction enzymes Sma I and Xma I. Annealing of these nucleotides into double-stranded structures leaves 4-base sticky ends that are compatible with those generated by Hind III on one end of the molecule (AGCT), and with Sal I-generated sticky ends on the other end of the molecule (TCGA). The sequence of these oligonucleotides is such that, when ligated onto the Hind III sticky ends, the recognition sequence for Hind III is destroyed.

DNA of pMSV A was digested with Hind III and Sal I, and was ligated to the above annealed oligonucleotides. An *E. coli* transformant harboring a plasmic containing the new oligonucleotides was identified by restriction enzyme site mapping, and was named **pMSV AB**.

3. **The C Block**: Complementary oligonucleotides having the sequences
CCGGGCCATTTGTTCCAGGCACGGGATAAGCATTCAGCCATGGGATATCAAGCTTGG
ATCCC (SEQ. ID. NO. 58) and
TCGAGGGATCCAAGCTTGATATCCCATGGCTGAATGCTTATCCCGTGCCTGGAACAA

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ATGGC (SEQ. ID. NO. 59) were synthesized and purified by standard procedures. The oligonucleotides incorporate bases that comprise recognition sites (underlined) for Nco I (CCATGG), EcoR V (GATATC), Hind III (AAGCTT), and BamH I (GGATCC). Annealing of these nucleotides into double-stranded structures leaves 4-base sticky ends that are compatible with those generated by Xma I on one end of the molecule (CCGG), and with Xho I-generated sticky ends on the other end of the molecule (TCGA). Such annealed molecules were ligated into **pMSV AB** DNA that had been digested with Xma I and Xho I. An *E.coli* transformant harboring a plasmid containing the oligonucleotide sequence was identified by restriction enzyme analysis, and DNA structure was verified by sequence analysis. The plasmid was named **pMSV CPL**; it contains the A, B and C blocks of nucleotides in sequential order ABC. Together, these comprise the 5' untranslated leader sequence ("L") of the MSV coat protein ("CP") gene. These correspond to nucleotides 167 to 186, and 188 to 317 of the MSV sequence of Mullineaux et al., (1984), and are flanked on the 5' end of the BamH I linker sequence GGATCCAG, and on the 3' end by the linker sequence GATATCAAGCTTGGATCCC (SEQ. ID. NO. 60). [Note: An A residue corresponding to base 187 of the wild type MSV sequence was inadvertently deleted during cloning.]

4. **Bgl II Site Insertion**: **pMSV CPL** DNA was digested at the Sma I site corresponding to base 277 of the MSV genomic sequence, and the DNA was ligated to Bgl II linkers having the sequence CAGATCTG. An *E.coli* transformant harboring a plasmic having a unique Bgl II site at the position of the former Sma I site was identified and verified by DNA sequence analysis, and the plasmid was named **pCPL-Bgl**.

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Example 7D

Construction of a deleted version of the maize alcohol dehydrogenase 1 (Adh1) intron 1

The starting material is plasmid **pVW119**, which was obtained from V. Walbot, Stanford University, Stanford, CA. This plasmid contains the DNA sequence of the maize Adh1.S gene, including intron 1, from nucleotides 119 to 672 [numbering of Dennis et al. (1984)], and was described in Callis et al. (1987). In pVW119, the sequence following base 672 of Dennis et al. (1984) is GACGGATCC, where the underlined bases represent a BamH I recognition site. The entire intron 1 sequence, with 14 bases of exon 1, and 9 bases of exon 2, can be obtained from this plasmid on a 556 bp fragment following digestion with Bcl I and BamH I.

1. Plasmid **pSG3525a(Pst)** DNA was digested with BamH I and Bcl I, and the 3430 bp fragment was purified from an agarose gel. [NOTE: The structure of plasmid **pSG3525a(Pst)** is not directly relevant to the end result of this construction series. It was constructed during an unrelated series, and was chosen because it contained restriction recognition sites for both Bcl I and BamH I, and lacks Hind III and Stu I sites. Those skilled in the art will realize that other plasmids can be substituted at this step with equivalent results.] DNA of plasmid pVW119 was digested with BamH I and Bcl I, and the gel purified fragment of 546 bp was ligated to the 3430 bp fragment. An *E.coli* transformant was identified that harbored a plasmid that generated fragments of 3430 and 546 upon digestion with BamH I and Bcl I. This plasmid was named **pSG AdhA1.**

2. DNA of **pSG AdhA1** was digested with Hind III, [which cuts between bases 209 and 210 of the Dennis et al., (1984) sequence, bottom strand], and with Stu I, which cuts between bases 554 and 555. The ends were made flush by T4 DNA polymerase treatment, and then ligated. An

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E.coli transformant that harbored a plasmid lacking Hind III and Stu I sites was identified, and the DNA structure was verified by sequence analysis. The plasmid was named **pSG AdhA1 Δ**. In this construct, 344 bp of DNA have been deleted from the interior of the intron 1. The loss of these bases does not affect splicing of this intron. The functional intron sequences are obtained on a 213 bp fragment following digestion with Bcl I and BamH I.

3. DNA of plasmid **pCPL-Bgl** (Example 7C Step 4), was digested with Bgl II, and the linearized DNA was ligated to the 213 bp Bcl I/BamH I fragment containing the deleted version of the Adh1.S intron sequences from **pSG AdhA1 Δ**. [Note: The sticky ends generated by digestion of DNA with Bgl II, Bcl I, and BamH I are compatible, but ligation of the BamH I or Bcl I sticky ends onto ones generated by Bgl II creates a sequence not cleaved by any of these three enzymes.] An *E.coli* transformant was identified by restriction enzyme site mapping that harbored a plasmid that contained the intron sequences ligated into the Bgl II site, in the orientation such that the Bgl II/Bcl I juncture was nearest the 5' end of the MSV CPL leader sequence, and the Bgl II/ BamH I juncture was nearest the 3' end of the CPL. This orientation was confirmed by DNA sequence analysis. The plasmid was named **pCPL A1I1 Δ**. The MSV leader/intron sequences can be obtained from this plasmid by digestion with BamH I and Nco I, and purification of the 373 bp fragment.

Example 7E

Construction of plant expression vectors based on the enhanced 35S promoter, the MSV CPL, and the deleted version of the Adh1 intron 1

1. DNA of plasmid **p35S En²/NOS** was digested with BamH I, and the 3562 bp linear fragment was ligated to a 171 bp fragment prepared from **pMSV CPL** DNA digested with BamH I. This fragment contains the entire MSV CPL sequence

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described in Example 7C. An *E.coli* transformant was identified by restriction enzyme site mapping that harbored a plasmid that contained these sequences in an orientation such that the Nco I site was positioned near the NOS Poly A sequences. This plasmid was named **p35S En² CPL/NOS**. It contains the enhanced version of the 35S promoter directly contiguous to the MSV leader sequences, such that the derived transcript will include the MSV sequences in its 5' untranslated portion.

2. DNA of plasmid **pKA882** (see Example 7B Step 1) was digested with Hind III and Nco I, and the large 4778 bp fragment was ligated to an 802 bp Hind III/Nco I fragment containing the enhanced 35S promoter sequences and MSV leader sequences from **p35S En² CPL/NOS**. An *E.coli* transformant harboring a plasmid that contained fragments of 4778 and 802 bp following digestion with Hind III and Nco I was identified, and named **pDAB310**. In this plasmid, the enhanced version of the 35S promoter is used to control expression of the GUS gene. The 5' untranslated leader portion of the transcript contains the leader sequence of the MSV coat protein gene.

3. DNA of plasmid **pDAB310** was digested with Nco I and Sac I. The large 3717 bp fragment was purified from an agarose gel and ligated to complementary synthetic oligonucleotides having the sequences CGGTACCTCGAGTTAAC (SEQ. ID. NO. 61) and CATGGTTAACTCGAGGTACCGAGCT (SEQ. ID. NO. 62). These oligonucleotides, when annealed into double stranded structures, generate molecules having sticky ends compatible with those left by Sac I, on one end of the molecule, and with Nco I on the other end of the molecule. In addition to restoring the sequences of the recognition sites for these two enzymes, new sites are formed for the enzymes Kpn I (GGTACC), Xho I (CTCGAG), and Hpa I (GTTAAC). An *E.coli* transformant was identified that harbored a plasmid that contained sites

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for these enzymes, and the DNA structure was verified by sequence analysis. This plasmid was named **pDAB1148**.

4. DNA of plasmid **pDAB1148** was digested with Bam HI and Nco I, the large 3577 bp fragment was purified from an agarose gel and ligated to a 373 bp fragment purified from **pCPL A1I1Δ** (Example 7D Step 3) following digestion with Bam HI and Nco I. An *E.coli* transformant was identified that harbored a plasmid with BamH I and Nco I, and the plasmid was named **pDAB303**. This plasmid has the following DNA structure: beginning with the base after the final G residue of the Pst I site of pUC19 (base 435), and reading on the strand contiguous to the coding strand of the *lacZ* gene, the linker sequence ATCTGCATGGGTG (SEQ. ID. NO. 63), nucleotides 7093 to 7344 of CaMV DNA, the linker sequence CATCGATG, nucleotides 7093 to 7439 of CaMV, the linker sequence GGGGACTCTAGAGGATCCAG (SEQ. ID. NO. 64), nucleotides 167 to 186 of MSV, nucleotides 188 to 277 of MSV, a C residue followed by nucleotides 119 to 209 of Adh1.S, nucleotides 555 to 672 of maize Adh1.S, the linker sequence GACGGATCTG, nucleotides 278 to 317 of MSV, the polylinker sequence GTTAACTCGAGGTACCGAGCTCGAATTTCCCC (SEQ. ID. NO. 65) containing recognition sites for Hpa I, Xho I, Kpn I, and Sac I, nucleotides 1298 to 1554 of NOS, and a G residue followed by the rest of the pUC19 sequence (including the EcoR I site). It is noteworthy that the junction between nucleotide 317 of MSV and the long polylinker sequence creates an Nco I recognition site.

5. DNA of plasmid **pDAB303** was digested with Nco I and Sac I, and the 3939 bp fragment was ligated to the 1866 bp fragment containing the GUS coding region prepared from similarly digested DNA of pKA882. The appropriate plasmid was identified by restriction enzyme site mapping, and was named **pDAB305**. This plasmid has the enhanced promoter, MSV leader and Adh1 intron arrangement

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of **pDAB303**, positioned to control expression of the GUS gene.

6. DNA of plasmid **pKA882** was digested with Xba I and Nco I and the 5687 bp fragment was ligated to annealed synthetic oligonucleotides having the sequence CTAGAGGATC (SEQ. ID. NO. 66) and CATGGATCCT (SEQ. ID. NO. 67). These oligonucleotides when annealed form a double-stranded structure having Xba I- and Nco I-compatible sticky ends. A recombinant plasmid lacking a Sal I site was identified by restriction enzyme mapping, verified by DNA sequence analysis, and was named **pDAB349.**

7. DNA of plasmid **p35S En²/NOS** was digested with Xba I and EcoR I, and the large fragment (3287 bp) was ligated to a 2152 bp fragment containing the GUS coding region and NOS polyadenylation region from similarly-digested pDAB349. A plasmid having the appropriate structure was identified by restriction site mapping, and was named **pDAB313**.

8. DNA of plasmid **pDAB313** was digested with Xba I and Sac I, and the large 3558 bp fragment was ligated to a 1889 bp fragment prepared from similarly-cut DNA of pKA882. A plasmid having the appropriate structure was identified by restriction site mapping and was named **pDAB348**.

9. DNA of plasmid **pDAB348** was digested with BamH I, and the large fragment (5437 bp) was ligated to a 213 bp Bcl I/BamH I fragment containing the deleted version of the Adh1.S intron 1, from pSG AdhAlΔ (Example 7D Step 2). A plasmid having the appropriate structure was identified by restriction site mapping and was named **pDAB353.**

EXAMPLE 7F

The starting material is plasmid pIC35. This plasmid contains the 845 bp *Sma I/Hind III* fragment from pUC13

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35S (-343) (see Section C of this example), ligated into the *Nru I* and *Hind III* sites of pIC19R (Marsh et al., Gene, 32 (1984) 481), in the orientation such that the *Hind III* recognition site is maintained. The source of the *A. tumefaciens* ORF25/26 sequences is plasmid pIC1925. This plasmid contains the 713 bp *Hinc II* fragment comprised by nucleotides 21728 to 22440 of *A. tumefaciens* pTi 15955 T-DNA (Barker et al., Plant Molec. Biol. 2 (1983) 335), ligated into the *Sma I* site of pIC19H (Marsh et al., Gene, 32 (1984) 481), in the orientation such that the *BamH I* site of pIC19H is adjacent to the ORF 25 end of the T-DNA fragment.

1. **pIC 19R35/A**: DNA of plasmid pIC35 was digested with *BamH I*, and ligated to the 738 bp fragment prepared by digestion of pIC1925 DNA with *BamH I* and *Bgl II*. An *E. coli* transformant was identified that harbored a plasmid in which a *BamH I* site was present positioned between the 35S promoter fragment, and the ORF 25/26 Poly A fragment. This plasmid was named **pIC 19R35/A**. (Note: Ligation of the compatible sticky ends generated by *BamH I* and *Bgl II* generates a sequence that is not a recognition site for either enzyme.)

2. **pIC35/A**: DNA of pIC 19R35/A was digested with *Sma I* at its unique site, and the DNA was ligated to *Bgl II* linkers having the sequence CAGATCTG. [NOTE: The tandomization of these *Bgl II* linkers generates, besides *Bgl II* recognition sites, also *Pst I* recognition sites, CTGCAG]. An *E. coli* transformant was identified that had at least two copies of the linkers (and therefore new *Bgl II* and *Pst I* sites) at the position of the former *Sma I* site. This plasmid was named **pIC35/A**.

3. **pIC 20RΔ**: DNA of plasmid pIC 20R (Marsh et al., Gene, 32 (1984) 481) was digested with *Nru I* and *Sma I*, and the blunt ends of the large fragment were ligated together. An *E. coli* transformant was identified that

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harbored a plasmid that lacked *Nru I*, *Sma I*, *Hind III*, *Sph I*, *Pst I*, *Sal I*, *Xba I*, and *BamH I* sites. This plasmid was called **pIC 20RΔ.**

4. **pSG Bgl 3525 (Pst)**: DNA of pIC 20R Δ was digested with *Bgl II*, and was ligated to the 1625 bp *Bgl II* fragment of pIC35/A. An *E. coli* transformant was identified that harbored a plasmid that contained the 35S promoter/ORF 25 poly A sequences. Restriction enzyme site mapping revealed these sequences to be in the orientation such that the unique *Kpn I* and *Xho I* sites of pIC 20RΔ are positioned at the 3' end of the ORF 25 Poly A sequences. This plasmid was named **pSG Bgl 3525 (Pst)**.

5. **pSG 3525 a (Pst)**: DNA of pSG Bgl 3525 (Pst) was digested with *Bgl II* under conditions in which only one of the two *Bgl II* sites of the molecule were cleaved. The 4301 bp linear fragments were ligated to synthetic adapter oligonucleotides having the sequence GATCGTGA TCAC (SEQ. ID. NO. 68), where the underlined bases represent the *Bcl I* recognition sequence. An *E. coli* transformant was identified that had a *Bcl I* site at the position of the former *Bgl II* site positioned 5' to the 35S promoter. This plasmid was named **pSG 3525 a (Pst)**.

6. **pDAB 218**: DNA of plasmid pIJ4104 (see Example 8) was digested with *Sma I*, and the 569 bp fragment was purified from an agarose gel. DNA of plasmid pSG 3525 a (Pst) (see above) was linearized by digestion at the unique *Hinc II* that lies between the 35S promoter and ORF 25 poly A sequences, and the linear fragment was ligated to the 569 bp *bar* gene fragment. An *E. coli* transformant was identified by restriction enzyme site mapping that harbored a plasmid that contained the *bar* gene in the orientation such that *Bgl II* digestion generated fragments of 4118 and 764 bp. This plasmid was named **pDAB 218.**

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7. **pDAB 219**: DNA of plasmid pDAB 218 was digested with *Bcl I*, and the linear fragment of 4882 bp was ligated to a 3133 bp *Bgl II* fragment prepared from DNA of pKA882-2xBg (see step 10 below). The latter fragment contains the GUS coding region, under the transcriptional control of the 35S promoter, with the Nos Poly A transcription termination signals. An *E. coli* transformant was identified that contained the GUS and PAT coding regions, and restriction enzyme recognition site mapping revealed that both coding regions were encoded by the same DNA strand. This plasmid was named **pDAB 219.**

8. DNA of plasmid pDAB 219 was used as the template for the polymerase chain reaction (PCR, (Saiki et al., Science, 239 (1988) 487)) using as primers the synthetic oligonucleotides: i) CTCGAGATCTAGATATCGATGAATTCCC (SEQ. ID. NO. 69), and ii) TATGGATCCTGTGATAACCGACATATGCCCCGGTTTCGTTG (SEQ. ID. NO. 70). Primer i) represents nucleotides 419 to 446 of pDAB 219, and includes bases corresponding to the recognition sites of *Xho I* (CTCGAG), *Bgl II* (AGATCT), *Xba I* (TCTAGA), *EcoR V* (GATATC), *Cla I* (ATCGAT), and *EcoR I* (GAATTC). The single underlined bases in Primer ii) represent the recognition sequence of *BamH I*, and the double underlined bases represent nucleotides 1138 to 1159 of pDAB 219, and correspond to nucleotides 21728 to 21749 of the ORF 25 Poly A fragment (see above). PCR amplification generated a product of 760 bp.

9. **pKA882-Bg**: pKA882 DNA was digested with *Pst I*, and the linear fragments were ligated to synthetic adaptors having the sequence CAGATCT GTGCA (SEQ. ID. NO. 71) (Note: When annealed, these molecules form double stranded molecules that have sticky ends compatible with those generated by *Pst I*. Ligation of such molecules to *Pst I* digested DNA results in a sequence that is no longer cleaved by *Pst I*, and introduces a new *Bgl II* site.). An *E. coli* transformant was identified that

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harbored a plasmid that was not cleaved by *Pst I*, and that had a unique *Bgl II* site. The plasmid was named pKA882-Bg.

10. **pKA882-2xBg**: pKA882-Bg DNA was digested with *EcoR I*, and the linear fragments were ligated to synthetic adaptors having the sequence AATTGAGATCTC (SEQ. ID. NO. 72). Ligation of annealed such molecules to *EcoR I* digested DNA results in a sequence that is no longer cleaved by *EcoR I*, and introduces a new *Bgl II* site. An *E. coli* transformant was identified that harbored a plasmid that was not cleaved by *EcoR I*, and that generated *Bgl II* fragments of 3027 and 2658 bp. This plasmid was named **pKA882-2xBg**.

11. **pDAB 305 Bg**: Plasmid pDAB305 was digested to completion with *EcoR I*, and the linearized DNA was ligated to kinased, self-complementary oligonucleotide adapters having the sequence AATTGAGATCTC (SEQ. ID. NO. 73). Ligation of this adapter to the overhanging ends generated by *EcoR I* recircularized the plasmid DNA, introduced a new *Bgl II* recognition site, and destroyed the former *EcoR I* recognition site. The resulting plasmid was named **pDAB 305 Bg**.

EXAMPLE 8: Construction of plant transformation vectors containing the *bar* gene of *Streptomyces hygroscopicus*

The starting material is plasmid pIJ4104 (White et al., Nucl. Acid Res. 18 (1990) 1062), which contains the coding region of the *bar* gene of *S. hygroscopicus*, and was obtained from M.J. Bibb (John Innes Institute, Norwich, United Kingdom). The *bar* gene encodes the enzyme phosphinothricin acetyl transferase (PAT).

pDAB 219Δ: DNA of plasmid pDAB 219 was digested with *Bgl II*, the 7252 bp fragment was purified from an agarose gel, and ligated to the 747 bp fragment

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generated by digestion of the PCR product of Example 7F Step 8 by *Bgl II* and *BamH I*. An *E. coli* transformant was identified that harbored a plasmid that contained a unique *Bgl II* site positioned at the 3' end of the ORF 25 Poly A fragment. The DNA structure of the 3' end of the PAT coding sequence was confirmed by DNA sequence analysis. This plasmid was named **pDAB 219Δ.**

The DNA sequence of pDAB 219Δ is as follows: Beginning with the base following the last A residue of the *Xba I* site on the lac Z coding strand of pIC20R (Marsh et al., Gene, 32 (1984) 481), the linker TCCTGATCTGTGCA GGTCCCC (SEQ. ID. NO. 74), followed by CaMV nucleotides 6605 to 7439, followed by the linker sequence GGGGACTCTAGAGGATCCGGATCCGTCGACCATGGTC (SEQ. ID. NO. 75), followed by the rest of the coding region of GUS with 44 bp of 3' flanking *E. coli* genomic DNA (nucleotides 306 to 2152 of Jefferson et al., (Proc. Natl. Acad. Sci., 83 (1986) 8447). The underlined bases represent the codons for the first two amino acids of the GUS protein, the second of which was changed from leucine in the original *E. coli* uid A gene (Jefferson et al., (Proc. Natl. Acad. Sci., 83 (1986) 8447) to valine in pRAJ275 (Jefferson et al., Plant Molec. Biol, Reporter, 5 (1987) 387). These bases are followed by the linker sequence GGGGAATTGGAGAGCTCGAATTTCCCC (SEQ. ID. NO. 76), then by bases 1298 to 1554 of the Nos Poly A sequence (DePicker et al., J. Molec. Appl. Genet., 1 (1982) 5561). The linker sequence GGGAATTGAGATCAGGATCTCGAGCTCGGG (SEQ. ID. NO. 77) is followed by bases 6495 to 6972 of CaMV, the linker CATCGATG, and CaMV bases 7090 to 7443. These bases are followed by the linker CAAGCTTGGCTGC AGGTC (SEQ. ID. NO. 78), then by bases corresponding to nucleotides 20 to 579 of the *bar* clone in pIJ4104 (White et al., Nucl. Acids Res. 18 (1990) 1062), the linker CTGTGATAACC (SEQ. ID. NO. 79), ORF 25/26 poly A nucleotides 21728 to 22440 (1), the linker GGGAATTCATCGATATCTAGATCTCGAGCTCGGGGTACCGAGCTCGAATTC (SEQ.

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ID. NO. 80) and the rest of pIC20R. The *Bgl II* recognition site (underlined) represents a unique site into which other genes may be introduced.

For expression in transgenic plant tissues and plants, the *Bt* ICP gene was subcloned into three different vectors. First, for cotransformation with plasmids carrying selectable and screenable markers, the ICP gene was cloned in plasmid pDAB305Bg. The *BamH I* site situated downstream of the ICP gene was modified to a *Sst I* site by insertion of a *BamH I/Sst I* adapter. The 1854 base pair *Nco I-Sst I* fragment carrying the ICP gene was inserted under the control of the high expression doubly enhanced 35S promoter and the nopaline synthase (Nos) poly A addition sequences, resulting in plasmid pDAB910 (Figure 6). Second, for transformation into MSD culture protoplasts and kanamycin selection, the enhanced 35S/Bt/Nos cassette was subcloned from pDAB910, as a 3150 base pair *Bgl II* fragment, into the unique *Bgl II* site of pDAB199, where the preparation of this plasmid is disclosed Sukhapinda et al.(Plant Cell Reports 13 (1993) 63), transformation of maize (Zea maysl) proplasts and regeneration resulting in plasmid pDAB911 (Figure 7). Third, the same 35SEn^2/Bt/Nos cassette was subcloned into the unique *Bgl II* site of pDAB219Δ, resulting in plasmid pDAB917 (Figure 8), for transformation by bombardment of TypeII callus and Basta™ selection.

EXAMPLE 9: Construction of a Reference Gene Encoding Firefly Luciferase.

Production of the GUS protein from genes controlled by different promoter versions was often compared relative to an internal control gene that produced firefly luciferase (DeWet et al., Molec. Cell Biol. 7 (1987) 725). A plasmid (pT3/T7-1 LUC) containing the luciferase (LUC) coding region was purchased from CLONTECH (Palo Alto, CA), and the coding region was

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modified at its 5' and 3' ends by standard methods. Briefly, the sequences surrounding the translational start (ATG) codon were modified to include an Nco I site (CCATGG) and an alanine codon (GCA) at the second position. At the 3' end, an Ssp I recognition site positioned 42 bp downstream of the Stop codon of the luciferase coding region was made blunt ended with T4 DNA polymerase, and ligated to synthetic oligonucleotide linkers encoding the Bgl II recognition sequence. These modifications permit the isolation of the intact luciferase coding region on a 1702 bp fragment following digestion by Nco I and Bgl II. This fragment was used to replace the GUS gene of plasmid pDAB305 (see Example 7E, step 5), such that the luciferase coding region was expressed from the enhanced 35S promoter, resulting in plasmid **pDeLux**. The 5' untranslated leader of the primary transcript includes the modified MSV leader/Adh intron sequence.

EXAMPLE 10: Cell Transformation.

Cell suspension cultures derived from immature maize microspores were used as the starting plant materials. These microspore-derived (MSD) cultures were maintained as described by Mitchell et al., J. Plant Physiol., 137 (1991) 530. The cultures are haploid, and some cell lines were capable of regenerating haploid plants. Eight- to 20-month old cell suspension cultures were used for protoplast isolation. The protoplast density was adjusted to 4 x 10^6 protoplasts/ml of electroporation solution [20 mg/L KH_2PO_4, 115 mg/L NaH_2PO_4, 444 mg/L $CaCl_2$, 7.5 g/L NaCl, 36.4 g/L mannitol, pH 7.2 (Fromm et al., Nature, 319 (1986) 791]. The protoplast suspension was heat shocked at 42°C for 5 minutes and then placed on ice. The plasmids pDAB 911 alone or pDAB 910 together with pDAB 326 were used in the protoplast transformation experiments. Equimolar DNA amounts of the plasmids (e.g. 64 µg of pDAB 911, 31.6 µg of pDAB 910 and

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46 µg of pDAB 326) were used. The plasmid DNA, in 20-40 µl sterile 1.0 mM Tris, pH 8.0, 1.0 mM EDTA, was placed in a one ml polystyrene electroporation cuvette containing a volume of the electroporation solution to make a total volume of 0.5 ml. One-half ml of the protoplast suspension was pipetted into the cuvette immediately before a single electrical pulse (400 µF, 300 v/cm) was applied from an IBI Gene Zapper unit. The cuvette was immediately placed on ice for 10 minutes. A volume of two hundred and fifty µl of the protoplast suspension (ca. 5 x 10^5 protoplasts) was spread on a filter (47 mm nylon; Micron Separations, Inc.) which was placed over the feeder cells (300 mg of MSD cells, Line 34) spread over M1 solid medium in a 60 x 15 mm polystyrene Petri plate. One week after plating, the filter was transferred to a selection medium containing 100 mg/L kanamycin sulfate. After four to six weeks on the kanamycin containing medium, resistant callus isolates could be observed and selected. From a total of four transformation experiments with the mentioned plasmids, over 400 isolates were selected. These callus isolates were grown on the same medium until enough tissue was accumulated for further analysis.

To determine whether these selected isolates were transformed and expressed the introduced marker genes, the callus tissues were assayed for ß-glucuronidase (GUS) activity using the histochemical technique described by Jefferson, Plant Molec. Biol. Rep., 5 (1987) 387, and for neomycin phosphotransferase (NPT II) activity using the technique described by Reiss et al., Gene, 30 (1984) 211. The selected isolates were tested for expression of the introduced ICP gene by immunoblot analysis as described above. The results are summarized in Table 16.

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Table 16. Summary of expression of ß-glucuronidase (GUS), neomycin phosphotransferase II (NPT II), and Bt insecticidal crystal protein (ICP) genes in transformed MSD calli.

Exp.#	Plasmid (s)	# Selected Isolates	# GUS[+]/ # Assayed	# NPT II[+]/ # Assayed	# ICP[a]/ # Assayed
1/28	pDAB 911	27	0/23	10/17	2/12
3/3	pDAB 911	117	5/117	13/21	0/20
3/6	pDAB 911 pDAB 910 + pDAB 326	95 98	0/95 30/98	1/26 8/12	NA 2/25
3/17	pDAB 911	105	0/105	4/7	0/8

A total of four isolates showed detectable levels of the ICP. Two isolates were transformed with pDAB 911, and their ICP expression level corresponds to approximately 0.1% of the total extractable protein (Figure 9). The other two isolates, obtained from cotransformation of pDAB 910 and pDAB 326, also expressed ICP at approximately 0.1% of total extractable protein (data not shown). Callus tissue from one isolate (transformed with pDAB 911) was used in a 3-day feeding assay of *Heliothis virescens* neonate larvae. The results (Table 17) indicated that the callus tissue produced sufficient ICP to kill most of the larvae, and severely inhibit the growth of survivors.

Table 17. Insecticidal activity of MSD callus, transformed with the ICP gene, in a 3-day *Heliothis virescens* feeding bioassay.

Exp. #	Diet	# Input Larvae	# Dead	Mean Mass of Survivors (mg)[b]	% Killed: (% Growth Inhibition)
1	MSD control	19	0	5.0 ± 0.55	0:(0)
2	MSD (ICP)	20	12[a]	0.26 ± 0.08	70; (95)

[a] 3 larvae escaped from the assay and were not counted. [b] ± 1 Standard Deviation.

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EXAMPLE 11: Cell Transformation

Part A - Establishment of Embryogenic Callus Cultures

Embryogenic callus cultures were initiated from immature embryos of genotypes specially bred for amenability to *in vitro* manipulation. Cultures representing two genotypes were used: i) "Backcrossed B73" is a BC_3 inbred derived from the cross B73x(B73xA188), and ii) "High II" is a hybrid made by intermating two S_3 lines derived from a B73xA188 cross. When exposed to appropriate cultural conditions, immature embryos from both of these genotypes display consistently high levels of callus formation capable of fertile plant regeneration.

Seeds of the two S_3 parents of "High II" and B73 were sown individually in pots containing approximately 4 kg of dry soil mix #3 (Conrad Fafard, Inc., Springfield, MA) moistened and adjusted to pH 6.0. The plants were maintained in a greenhouse under a 16/8 photoperiod. Ambient daylight was supplemented with a combination of high pressure sodium and metal halide lamps such that the minimum light intensity 2 m above pot level was approximately 1,500 ft-candles. Greenhouse temperature was maintained within 3°C of 28°C during the day and 22°C at night. The plants were irrigated as needed with a solution containing 400 mg/L of 20-20-20 fertilizer (W.R. Grace & Co., Fogelsville, PA), plus 8 mg/L chelated iron (CIBA-GEIGY, Greensboro, NC).

Pollen shed and silk emergence began 50-60 days after planting. Female plants were prepared for pollination on the day before pollen availability by cutting off the tip of the husks and silks of unfertilized ear shoots. The next day, after the silks had grown to form a thick "brush" all the same length, pollen was collected by placing paper bags over the

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tassels and carefully applied to the silks. "Backcrossed B73" embryos were produced on B73 plants by pollinating with plants regenerated from BC_2 cultures (as described below). "High II" embryos resulted from intermating the S_3 lines.

When the developing embryos reached a length of approximately 1.5-2.0 mm (10-14 days after pollination), the ear was excised and surface sterilized by emersion in 70% v/v ethanol for 10 minutes followed by soaking in 20% v/v commercial bleach (1% sodium hypochlorite) for 30 minutes. Following a sterile, distilled water rinse, immature embryos were aseptically isolated and placed onto an "initiation" medium with the embryo axis in contact with the medium (scutellar-side away from the medium). The "initiation" medium consisted of the following components: N6 basal salts and vitamins (Chu, Proc. Symp. Plant Tissue Culture, (1978), Peking Press, pp. 43-56), 20 g/L sucrose, 2.9 g/L proline, 100 mg/l casein hydrolysate, 1 mg/l 2,4-dichloro-phenoxyacetic acid (2,4-D), 10 mg/L $AgNO_3$ and 2.5 g/L gelrite (Kelco, Inc., San Diego, CA) adjusted to pH 5.8.

The immature embryos were incubated at 28°C in the dark for 10-30 days during which time callus tissue, displaying various types of morphology, proliferated from the scutellar region. The callus tissue produced during this time was classified into three distinct types: i) soft, granular, translucent callus lacking any apparent morphological organization (known as nonembrogenic), ii) compact, nodular, yellowish-to-white callus consisting of groups of somatic embryos (often fused) with distinct scutellar- and coleoptile-like structures (known as Type I), and iii) soft callus with numerous globular and elongated somatic embryos on suspensor-like structures (known as Type II). Type II callus was the most suitable for establishing friable, embryogenic cultures. Sometimes entire scutella proliferated with this type of

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tissue or at times only small sectors exhibiting this morphology developed. Selective subculture was then performed, whereby only tissue with well-defined globular and elongated somatic embryos along with some subtending undifferentiated, soft tissue was transferred to fresh "initiation" medium. After 2-3 subcultures on "initiation" medium, callus was transferred to "maintenance" medium. The "Maintenance" medium differed from the "initiation" medium in that it contained 690 mg/L proline and no $AgNO_3$. After 8-16 weeks of preferential enrichment for Type II callus, well-established, embryogenic cultures were ready for helium blasting.

Part B - Transformation via Helium Blasting

Helium blasting involved accelerating micron-size particles, coated with plasmid DNA, to penetrating velocities. The device used was described in U.S. Patent No. 5,141,131. Briefly, the device consisted of a high pressure helium source, a reservoir of DNA-coated gold microparticles in suspension, and a multipurpose valve which provided selective communication between the outlet of the helium source and the inlet of the gold suspension. The gold particles were coated with plasmid DNA (pDAB917) containing coding sequences for selectable and screenable marker genes.

The selectable marker gene was *bar* which encodes for the enzyme phosphinothricin acetyltransferase (PAT) and confers resistance to the herbicide Basta™. The screenable marker gene was *uidA* which encodes for β-glucuronidase (GUS), the activity of which was monitored histochemically. Both genes were driven by the 35S constitutive promoter from Cauliflower Mosaic Virus. In this way, rare transformed cells were selected out of a background of non-transformed tissue by exposure to the herbicide Basta™ and tested for the presence of β-

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glucuronidase activity using a histochemical assay which turned positive tissue blue.

Plasmid DNA was adsorbed onto the surface of gold particles prior to use in transformation experiments. The gold particles were spherical with diameters ranging from about 1.5-3.0 microns (Aldrich Chemical Co., Milwaukee, WI). Adsorption was accomplished by adding 74 uL of 2.5 M calcium chloride and 30 uL of 0.1 M spermidine to 300 uL of DNA/gold suspension (140 ug pDAB917, 0.01 M Tris buffer, and 1mM EDTA). The DNA-coated gold particles were vortexed immediately, then allowed to settle to the bottom of an Eppendorf tube and the resultant clear liquid was completely drawn off. The DNA-coated gold particles were then resuspended in 1 mL of 100% ethanol. The suspension was then diluted to 15 mg DNA/gold per mL of ethanol for use in helium blasting experiments.

Approximately 250 mg of embryogenic callus tissue, 5-7 days following subculture, was arranged in a thin circular layer directly on the surface of "maintenance" medium. The tissue was allowed to dry out slightly by allowing the plates to stand uncovered in a laminar flow hood for several minutes before use. In preparation for helium blasting, the callus was covered with a 104 micron stainless steel screen. The DNA-coated gold particles were then accelerated at the callus tissue. Each callus tissue sample was blasted 10-15 times with each blast delivering approximately 1 uL of DNA-coated gold suspension.

Part C - Selection of Transgenic Tissue and Plant Regeneration

After blasting, callus tissue was allowed to incubate for 1-2 days under the conditions described previously. Each tissue sample was then divided into

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approximately 60 equal pieces (1-3 mm diameter) and transferred to fresh "maintenance" medium containing 30 mg/L Basta™. Every three weeks, callus tissue was non-selectively transferred (with no regard for tissue morphology) to fresh Basta™-containing "maintenance" medium. At this concentration of herbicide, very little growth occurred. After 8-16 weeks, sectors proliferating from a background of growth inhibited tissue were apparent. This tissue was isolated from the other callus and maintained separately on Basta™-containing "maintenance" medium and selectively subcultured (only Type II tissue) every 10-14 days. At this point, a histochemical assay for GUS expression was performed as described below.

All Basta™-resistant callus (whether GUS positive or GUS negative) was selectively subcultured to "induction" medium and incubated at 28°C in low light (125 ft-candles) for one week followed by one week in high light (325 ft-candles) provided by cool fluorescent lamps. The "induction" medium was composed of MS salts and vitamins (Murashige et al., Physiol. Plant, 15 (1962) 473-497) 30 g/L sucrose, 100 mg/L myo-inositol, 5 mg/L benzyl-amino purine, 0.025 mg/L 2,4-D, 2.5 g/L gelrite adjusted to pH 5.7. Following this two-week induction period, the callus was then non-selectively transferred to "regeneration" medium and incubated in high light at 28°C.

The "regeneration" medium was composed of MS salts and vitamins, 30 g/L sucrose, and 2.5 g/L gelrite adjusted to pH 5.7. Every 14-21 days the callus was subcultured to fresh "regeneration" medium selecting for tissue which appeared to be differentiating leaves and roots. Both "induction" and "regeneration" media contained 30 mg/L Basta™. Plantlets were transferred to 10 cm pots containing approximately 0.1 kg of dry soil mix, and were then moistened thoroughly and covered with

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clear plastic cups for approximately 4 days. At the 3-5 leaf stage, plants were transplanted to larger pots and grown to maturity as previously described. Self- or sibling-pollinations were performed on plants regenerated from the same culture or crossed to non-transformed seed-derived plants in order to obtain transgenic progenies.

EXAMPLE 12: Field Trials

Using the procedures and transgenic progency described in Example 11, four (4) transgenic inbreds were prepared using conventional breeding techniques. The resulting inbreds were used to develop four transgenic hybrids.

Seed from each of four (4) transgenic hybrids were planted in single row plots using a randomized complete block design. Locations included research stations in Indiana, Illinois, Minnesota, and Iowa. Control plots (non-transgenic control hybrids) were used to measure the amount of insect damage due to natural (Control A) and artificial infestations (Control B). Control of second generation European Corn Bore (ECB) was evaluated at all locations. First generation ECB and corn earworm were evaluated only at the Indiana and Illinois field research stations. All insects were obtained from a single source. Each trial was infested twice (4-6 days apart) with neonate larvae. For first generation ECB studies, 40-80 larvae were applied to plants at the mid-whorl development stage, while the same number of larvae were applied at mid-silk stage in second generation ECB studies. Damage to plants was determined 6 weeks later by splitting stalks and ear shoots when present. Number of ECB larvae and tunnels were recorded for each of 10 plants per replicate. Studies on corn earworm required 10 plants per replicate to be artificially infested with first instar larvae of corn earworm at about 5-10 per ear. Approximately 3 weeks

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later, ears were evaluated for the number of larvae present.

A combined analysis of variance was conducted on data collected for first generation ECB studies (Table 18). Artificially infested controls averaged one tunnel per stalk and had a level of infestation over 70 percent. Transgenic lines showed little to no ECB tunneling ($\leq$0.06 tunnels per stalk) and had levels of infestation below 7 percent. A significant difference ($p<0.05$) was shown between controls and transgenic lines for larvae and tunnels per stalk as well as the percentage of infested plants. No statistical differences were found between the individual transgenic hybrids for control of first generation ECB.

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Table 18. First Generation ECB Data.

	Stalk			Ear	
INDIANA	Number of Larvae	Number of Tunnels	% Stalk Infested	Number of Larvae	Number of Tunnels
Control A	0.05	0.33	25.0	n/a	n/a
Control B	0.25	1.23	72.5	n/a	n/a
Hybrid 1	0.00	0.00	0.0	n/a	n/a
Hybrid 2	0.00	0.00	0.0	n/a	n/a
Hybrid 3	0.00	0.00	0.0	n/a	n/a
Hybrid 4	0.00	0.03	2.5	n/a	n/a
ILLINOIS					
Control A	0.20	0.30	27.5	0.08	0.05
Control B	0.54	1.06	74.8	0.21	0.13
Hybrid 1	0.00	0.00	0.0	0.00	0.00
Hybrid 2	0.00	0.03	2.5	0.00	0.00
Hybrid 3	0.00	0.13	12.5	0.00	0.00
Hybrid 4	0.00	0.00	0.0	0.00	0.00
Combined Analysis					
Control A	0.13	0.31	26.3		
Control B	0.40	1.14	73.6		
Hybrid 1	n/a	n/a	n/a		
Hybrid 2	0.00	0.01	1.3		
Hybrid 3	0.00	0.06	6.3		
Hybrid 4	0.00	0.01	1.3		

For second generation ECB, artificially infested controls averaged between 1 to 3 tunnels per stalk; levels of infestation ranged from 72 to 100 percent (Table 19). Damage to transgenic hybrids ranged from none to slight ($\leq$ 0.25 tunnels per stalk) with levels of infestation that ranged from 0 to 23 percent (Table 19). Measurements made on tunnel length showed that tunnels found in the transgenic lines were significantly smaller ($p < 0.5$) compared to the controls

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(Table 19). Only mean and standard error of the mean were calculated for average tunnel length measurement; other statistical analyses were invalid because the lack of tunnels in the many replicates of the transgenics resulted in missing data. With the exception of the study in Minnesota, these data show that average tunnel length among transgenic hybrids were similar and smaller than the controls. Transgenic lines had significantly less damaged ears from ECB ($p < 0.05$) than the controls. In general, significant differences ($p < 0.05$) were found between controls and the transgenic lines. No statistically significant differences were detected between individual transgenic hybrids and their level of control to second generation ECB.

Table 19. Second Generation ECB Data.

	Ear		Stalk				Average Tunnel Length *	
INDIANA	# of Larvae	# of Tunnels	# of Larvae	# of Tunnels	Total Tunnel Length	% Stalks Infested	Mean	S.E. of Mean
Control A	0.18	0.10	0.28	0.35	1.05	47.5	2.92	± 0.61
Control B	0.71	0.77	1.24	1.59	3.78	94.8	2.38	± 0.20
Hybrid 1	0.03	0.00	0.00	0.00	0.00	2.5	n/a	n/a
Hybrid 2	0.00	0.00	0.00	0.00	0.00	0.0	n/a	n/a
Hybrid 3	0.00	0.00	0.03	0.03	0.03	2.5	1.00	n/a
Hybrid 4	0.00	0.00	0.00	0.00	0.00	0.0	n/a	n/a
ILLINOIS								
Control A	0.45	0.60	1.60	1.83	9.21	85.0	5.85	± 0.84
Control B	0.58	0.68	2.20	2.55	11.11	100.0	4.38	± 0.39
Hybrid 1	0.03	0.03	0.00	0.05	0.13	5.0	2.50	n/a
Hybrid 2	0.00	0.00	0.00	0.08	0.19	5.0	2.53	± 0.02
Hybrid 3	0.00	0.00	0.00	0.13	0.32	10.0	2.52	± 0.01
Hybrid 4	0.00	0.00	0.00	0.08	0.19	7.5	2.50	± 0.00
IOWA								
Control A	0.13	0.23	0.68	1.80	3.35	82.5	2.06	± 0.15
Control B	0.15	0.25	0.73	1.90	4.85	95.0	2.37	± 0.23
Hybrid 1	0.00	0.03	0.03	0.25	0.42	22.5	1.58	± 0.35

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Hybrid 2	0.00	0.00	0.00	0.13	0.18	10.0	1.38	± 0.43
Hybrid 3	0.00	0.00	0.00	0.13	0.10	12.5	0.80	± 0.12
Hybrid 4	0.00	0.00	0.00	0.13	0.10	12.5	0.80	± 0.12
MINNESOTA								
Control A	0.00	0.05	0.40	0.68	1.79	47.5	2.63	± 0.58
Control B	0.03	0.16	0.67	1.43	3.39	71.8	2.61	± 0.38
Hybrid 1	0.00	0.00	0.03	0.03	0.08	2.5	3.00	n/a
Hybrid 2	0.00	0.00	0.00	0.03	0.04	2.5	1.50	n/a
Hybrid 3	0.00	0.00	0.00	0.03	0.01	2.5	0.50	n/a
Hybrid 4	0.00	0.00	0.03	0.05	0.15	5.0	3.00	± 1.00
Combined Analyses								
Control A	0.19	0.24	0.74	1.16	3.85	65.6		
Control b	0.37	0.46	1.21	1.87	5.78	90.4		
Hybrid 1	0.01	0.01	0.01	0.08	0.16	8.1		
Hybrid 2	0.00	0.00	0.00	0.06	0.10	4.4		
Hybrid 3	0.00	0.00	0.01	0.08	0.11	6.9		
Hybrid 4	0.00	0.00	0.01	0.06	0.11	6.3		

Artificially infested controls averaged approximately one earworm larvae per ear and a range of infestation between 40 and 90 percent. Transgenic hybrids were significantly different ($p < 0.05$) from controls for both earworm per ear and percent plants infested. Although no statistically significant difference between transgenic hybrids was detected, hybrid #1 showed damage from earworm at both locations (Table 20). Hybrids 2, 3 and 4 showed little to no damage from the insect.

Table 20. Corn Earworm Data.

INDIANA	# of Earworm	% Infested	# of ECB	% Infested
Control A	0.15	15.0	n/a	n/a
Control B	1.18	80.0	n/a	n/a
Hybrid 1	0.05	5.0	n/a	n/a
Hybrid 2	0.00	0.0	n/a	n/a

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Hybrid 3	0.00	0.0	n/a	n/a
Hybrid 4	0.00	0.0	n/a	n/a
ILLINOIS				
Control A	0.23	17.5	0.28	25.0
Control B	1.12	58.0	0.27	23.8
Hybrid 1	0.28	27.5	0.03	2.5
Hybrid 2	0.03	2.5	0.00	0.0
Hybrid 3	0.00	0.0	0.00	0.0
Hybrid 4	0.00	0.0	0.00	0.0
Combined Analysis				
Control A	0.19	16.3		
Control B	1.15	69.0		
Hybrid 1	0.16	16.3		
Hybrid 2	0.01	1.3		
Hybrid 3	0.00	0.0		
Hybrid 4	0.00	0.0		

Numerous modifications and variations in practice of the invention are expected to occur to those skilled in the art upon consideration of the foregoing detailed description of the invention. Consequently, such modifications and variations are intended to be included within the scope of the following claims.

EXAMPLE 13: Determination of Relative Promoter Strengths by Transient Expression in Electroporated

Black Mexican Sweet (BMS) cultures (V. Walbot, Stanford University) were maintained as suspensions in liquid medium (Fromm et al., PNAS USA 82 (1985) 351). Protoplasts were isolated from 4-day old cultures by suspending the cells in 4X volumes of protoplast isolation solution (Fromm et al., Enzymol. 153 (1987) 351) containing 0.5% cellulase Onozuka RS, 0.5% hemicellulase, 0.02% pectinase (Karlan Research Products, Santa Rosa, CA), followed by gentle shaking. After 3.5

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hr digestion, cells and protoplasts were collected by centrifugation, (208 x g, 25°C, 5 min), and washed twice by gentle resuspension in protoplast isolation solution. Purification of protoplasts was achieved by flotation on Maize Wash Solution (Shanin, Theor. Appl. Genet. 69 (1985) 235). Protoplasts were washed twice in electroporation solution (Fromm et al., Enzymol. 153 (1987) 351), and brought to a final density of 4 x 10^6 protoplasts/ml. Prior to electroporation, the protoplasts were heat shocked for 5 min at 42°C, then placed on ice until use. Aliquots of about 2 x 10^6 protoplasts were electroporated with the appropriate DNA mixture in a 1 ml volume. Typically DNA mixtures contained (per 2 x 10^6 protoplasts in 1 ml), 60 ug of test plasmid DNA and 4.5 ug of reference plasmid DNA. Electroporation conditions were: 1500 uF, 200-400V across a 1 cm gap, pulse time of 25 msec (Promega Model 240/250, Madison, WI). Following electroporation, the protoplasts were placed on ice for 10 min, then plated into plastic Petri dishes (previously coated with a thin layer of 1.2% SeaPlaque agarose; FMS BioProducts, Rockland, ME) containing protoplast growth medium (Fromm et al., PNAS USA 82 (1985) 351) at a density of 2.5 x 10^5 protoplasts/ml.

Fluorometric assays for GUS activity using 4-methyl-umbelliferyl-glucuronide as a substrate were essentially as described by Jefferson (Plant Molec. Biol. Reporter 5 (1987) 387), and assays for luciferase activity using luciferin as substrate were based on the methods of DeWet et al. (Molec. Cell. Biol. 7 (1987) 725), Ow et al. (Science 234 (1986) 856), Ow et al. (PNAS USA 84 (1987) 4870), and Howell et al., (Plant Molecular Biology Manual (1989) Ch. B8,1). In some cases the GUS and LUC genes were coelectroporated on separate plasmids, in others they were introduced on a single plasmid.

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The results of comparative promoter strength studies are given below.

Table 21.

Plasmid	Promoter	5' UTL*	Relative Strength
pKA882	35S	Linker A	1
pDAB348	En 35S	Linker A	0.8
pDAB310	En 35S	MSV CPL	0.1
pDAB353	En 35S	Adh1.S intron1Δ	5.8
pDAB305	En 35S	MSV CPL + Adh1.S intron1Δ	42

*UTL = Untranslated Leader Sequence

These data demonstrate that no expression advantage is gained by duplication of the 35S enhancer element in maize protoplasts, nor does the MSV coat protein leader sequence confer a translational enhancement by itself. Some expression enhancement is seen when the deleted version of the maize Adh1.S intron 1 is positioned within the 5' untranslated leader. However, an approximately 40-fold increase in GUS expression over the native 35S promoter is observed when the enhanced 35S promoter is coupled to the MSV leader containing the deleted version of the Adh1.S intron 1. The sequence of the promoter/leader combination is listed as SEQ. ID. NO. 43.

EXAMPLE 14: Cloning of Intron 6

This example describes the cloning of intron 6 of the maize Adh1.S gene and its incorporation into the synthetic 5' untranslated leader sequence derived from

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the Maize Streak Virus coat protein gene (MSV/CPL, see above).

The starting material is plasmid pB428, obtained from J. Bennetson, Purdue University. This is a clone if ab 11.5 kbp BamH I fragment of maize genomic DNA inserted into the BamH I site of pBR322, and containing the Adh1.S gene (Dennis et al., Nucl. Acids Res. 12 (1984) 3983). A 396 bp fragment containing the intron 6 sequence and parts of flanking exons 6 and 7 was amplified from 10 ng of pB428 template DNA using 100 pmol each of forward primers having the sequence CGACCTGATCACCCCAGCAGATTCGAAGAAGG (SEQ. ID. NO. 81), and reverse primers of sequence TTCAGTGGATCCGAACTTCCTAGCTGAAAAATGGG (SEQ. ID. NO. 82). These primers contain the recognition sequences for Bcl I (TGATCA, underlined in forward primer), and BamH I, (GGATCC, underlined in reverse primers). They are designed to introduce the Bc I site immediately before nucleotide 2162, and the BamH I site immediately following nucleotide 2534, of the Adh1.S sequence of Dennis et al. (Nucl. Acids Res. 12 (1984) 3983). The resulting PCR fragment, of expected size 396 bp, contains 20 bases of Adh1.S exon 6, all of intron 6, and 11 bases of exon 7, as presented in (SEQ. ID. NO. 83)

Reactions (100 ul final volume) contained, besides template and primers, 1 x PCR reaction buffer (as described in Example 2), 0.2 mM final concentration of dATP, dTTP, dGTP, and dCTP, and 5 units of Taq DNA polymerase (Perkin Elmer/Cetus). Temperature cycles were: 94° (1 min; 25 cycles of 94° (1 min), 55° (30 sec), 72° (30 sec), followed by an extension period of 72°, 10 min. Appropriate-sized fragments were extracted from an agarose gel, digested with restriction enzymes Bcl I and BamH I, and ligated into Bgl II-digested DNA of pCPL-Bg (see above). A plasmid was identified that had an appropriate restriction enzyme map, and was named pCPL-Adh6.

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The structure of pCPL-Adh6 is as follows (vector sequences of pBSK are not included, see Example 7C step 1): the linker sequence GGATCCAG that includes a BamH I recognition site, nucleotides 167 to 186 of MSV, nucleotides 188 to 277 of MSV, the linker sequence GATCA, nucleotides 2162 to 2534 of maize Adh1.S, the linker sequence GGATCTG, and finally nucleotides 278 to 317 of MSV, including an Nco I recognition sequence (SEQ. ID. NO. 84). In analogy to pCPL AlI1Δ (see Example 7D step 3) the MSV leader/intron sequences can be obtained from this plasmid by digestion with BamH I and Nco I, and purification of the 541 bp fragment. This fragment is therefore the functional equivalent of the analogous fragment containing the Adh1.S intron 1 fragment utilized in palsmids described in Examples 7 and 13.

The nucleotide sequence coding for an insecticial protein from *Bt* having the nucleotide SEQ. ID. NO. 1 and amino acid SEQ. ID. NO. 2 is shown in Table 22.

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SEQ. ID. NO. 1 and 2

```
1    ATG GAC AAC AAC CCA AAC ATC AAC GAG TGC ATC CCT TAC AAC TGC TTG AGC AAC CCT GAG GTG GAG GTG CTT GGT GGT GAG CGC ATT GAG
1    Met Asp Asn Asn Pro Asn Ile Asn Glu Cys Ile Pro Tyr Asn Cys Leu Ser Asn Pro Glu Val Glu Val Leu Gly Gly Glu Arg Ile Glu
91   ACC GGC TAC ACC CCA ATC GAC ATC TCT CTG AGC CTG ACC CAA TTC CTG CTG TCT GAG TTC GTG CCA GGT GCT GGC TTC GTG CTT GGT CTG
31   Thr Gly Tyr Thr Pro Ile Asp Ile Ser Leu Ser Leu Thr Gln Phe Leu Leu Ser Glu Phe Val Pro Gly Ala Gly Phe Val Leu Gly Leu
                SalI
181  GTC GAC ATC ATC TGG GGC ATC TTC GGC CCA AGC CAA TGG GAC GCT TTC CTT GTG CAA ATC GAG CAG CTC ATC AAC CAA CGC ATT GAG GAG
61   Val Asp Ile Ile Trp Gly Ile Phe Gly Pro Ser Gln Trp Asp Ala Phe Leu Val Gln Ile Glu Gln Leu Ile Asn Gln Arg Ile Glu Glu
271  TTC GCT CGC AAC CAA GCC ATC TCC AGG CTT GAG GGC CTG AGC AAC CTG TAC CAA ATC TAC GCT GAG AGC TTC AGG GAG TGG GAG GCT GAC
91   Phe Ala Arg Asn Gln Ala Ile Ser Arg Leu Glu Gly Leu Ser Asn Leu Tyr Gln Ile Tyr Ala Glu Ser Phe Arg Glu Trp Glu Ala Asp
361  CCA ACC AAC CCA GCC CTG AGG GAG GAG ATG CGC ATC CAA TTC AAC GAC ATG AAC TCT GCT CTG ACC ACG GCC ATC CCT CTG TTC GCT GTG
121  Pro Thr Asn Pro Ala Leu Arg Glu Glu Met Arg Ile Gln Phe Asn Asp Met Asn Ser Ala Leu Thr Thr Ala Ile Pro Leu Phe Ala Val
451  CAG AAC TAC CAA GTG CCT CTT CTG AGC GTC TAC GTG CAA GCT GCC AAC TTG CAC CTG TCT GTG TTG AGG GAC GTG TCT GTG TTC GGT CAA
151  Gln Asn Tyr Gln Val Pro Leu Leu Ser Val Tyr Val Gln Ala Ala Asn Leu His Leu Ser Val Leu Arg Asp Val Ser Val Phe Gly Gln
541  CGC TGG GGC TTC GAT GCT GCC ACC ATC AAC TCT CGC TAC AAC GAC CTG ACC AGG CTG ATT GGC AAC TAC ACG GAC TAC GCT GTG CGC TGG
181  Arg Trp Gly Phe Asp Ala Ala Thr Ile Asn Ser Arg Tyr Asn Asp Leu Thr Arg Leu Ile Gly Asn Tyr Thr Asp Tyr Ala Val Arg Trp
                XhoI
631  TAC AAC AGG GGC CTC GAG AGG GTG TGG GGT CCA GAC TCC AGG GAC TGG GTG CGT TAC AAC CAA TTC CGC AGG GAG CTG ACC CTG ACG GTG
211  Tyr Asn Thr Gly Leu Glu Arg Val Trp Gly Pro Asp Ser Arg Asp Trp Val Arg Tyr Asn Gln Phe Arg Arg Glu Leu Thr Leu Thr Val
721  CTT GAC ATT GTG GCT CTG TTC CCA AAC TAC GAC TCC CGT CGC TAC CCA ATC CGC ACG GTG TCT CAG CTG ACT AGG GAG ATT TAC ACC AAC
241  Leu Asp Ile Val Ala Leu Phe Pro Asn Tyr Asp Ser Arg Arg Tyr Pro Ile Arg Thr Val Ser Gln Leu Thr Arg Glu Ile Tyr Thr Asn
811  CCA GTT TTG GAG AAC TTC GAT GGC TCC TTC AGG GGC TCT GCT CAA GGC ATT GAG CGC AGC ATT CGC TCT CCT CAC CTG ATG GAC ATT CTT
271  Pro Val Leu Glu Asn Phe Asp Gly Ser Phe Arg Gly Ser Ala Gln Gly Ile Glu Arg Ser Ile Arg Ser Pro His Leu Met Asp Ile Leu
901  AAC AGC ATC ACT ATC TAC ACG GAC GCT CAC CGC GGC TAC TAC TAC TGG TCT GGC CAC CAA ATC ATG GCT TCC CCA GTT GGT TTC TCT GGC
301  Asn Ser Ile Thr Ile Tyr Thr Asp Ala His Arg Gly Tyr Tyr Tyr Trp Ser Gly His Gln Ile Met Ala Ser Pro Val Gly Phe Ser Gly
991  CCA GAG TTC ACC TTC CCT TTG TAC GGT ACG ATG GGC AAC GCT GCT CCA CAA CAG CGC ATT GTG GCT CAA CTG GGC CAA GTT GTG TAC CGC
331  Pro Glu Phe Thr Phe Pro Leu Tyr Gly Thr Met Gly Asn Ala Ala Pro Gln Gln Arg Ile Val Ala Gln Leu Gly Gln Gly Val Tyr Arg
1081 ACC CTT TCC AGC ACC TTG TAC CGC CGC CCA TTC AAC ATT GGC ATC AAC AAC CAA CAG CTT TCT GTG CTT GAT GGC ACT GAG TTC GCT TAC
361  Thr Leu Ser Ser Thr Leu Tyr Arg Arg Pro Phe Asn Ile Gly Ile Asn Asn Gln Gln Leu Ser Val Leu Asp Gly Thr Glu Phe Ala Tyr
                                                    XpaI
1171 GGC ACC TCT AGC AAC TTG CCA ATC GCT GTG TAC CGC AAG TCT GGT ACC GTG GAC AGC TTG GAC GAG ATC CCT CCA CAG AAC AAC AAC GTG
391  Gly Thr Ser Ser Asn Leu Pro Ser Ala Val Tyr Arg Lys Ser Gly Thr Val Asp Ser Leu Asp Glu Ile Pro Pro Gln Asn Asn Asn Val
1261 CCA CCT CGC CAA GGC TTC TCT CAC CGC TTG AGC CAC GTT TCC ATG TTC CGT TCG GGC TTC AGC AAC AGC TCT GTG AGC ATT ATC AGG GCC
421  Pro Pro Arg Gln Gly Phe Ser His Arg Leu Ser His Val Ser Met Phe Arg Ser Gly Phe Ser Asn Ser Ser Val Ser Ile Ile Arg Ala
1351 CCA ATG TTC TCC TGG ATT CAC CGT TCT GCT GAG TTC AAC AAC ATC ATT GCC TCT GAC AGC ATC ACT CAA ATC CCT GCT GTT AAG GGC AAC
451  Pro Met Phe Ser Trp Ile His Arg Ser Ala Glu Phe Asn Asn Ile Ile Ala Ser Asp Ser Ile Thr Gln Ile Pro Ala Val Lys Gly Asn
1441 TTC CTT TTC AAC GGC TCG GTC ATC TCG GGT CCA GGT TTC ACG GGT GGT GAC CTT GTG CGC TTG AAC AGC TCG GGT AAC AAC ATC CAG AAC
481  Phe Leu Phe Asn Gly Ser Val Ile Ser Gly Pro Gly Phe Thr Gly Gly Asp Leu Val Arg Leu Asn Ser Ser Gly Asn Asn Ile Gln Asn
1531 AGG GGT TAC ATC GAG GTG CCA ATC CAC TTC CCA TCC ACC AGC ACC CGC TAC AGG GTC AGG GTC AGG TAC GCT TCT GTG ACC CCA ATC CAC
511  Arg Gly Tyr Ile Glu Val Pro Ile His Phe Pro Ser Thr Ser Thr Arg Tyr Arg Val Arg Val Arg Tyr Ala Ser Val Thr Pro Ile His
                                                                PstI
1621 TTG AAC GTG AAC TGG GGC AAC AGC TCC ATC TTC AGC AAC ACG GTG CCA GCC ACG GCC ACC AGC CTT GAC AAC CTG CAG TCC TCG GAC TTC
541  Leu Asn Val Asn Trp Gly Asn Ser Ser Ile Phe Ser Asn Thr Val Pro Ala Thr Ala Thr Ser Leu Asp Asn Leu Gln Ser Ser Asp Phe
1711 GGC TAC TTC GAA TCG GCC AAC GCT TTC ACC AGC TCT CTT GGC AAC ATC GTG CGT GTG CGC AAC TTC TCT GGC ACG GCT GGT GTC ATC ATC
571  Gly Tyr Phe Glu Ser Ala Asn Ala Phe Thr Ser Ser Leu Gly Asn Ile Val Gly Val Arg Asn Phe Ser Gly Thr Ala Gly Val Ile Ile
                EcoRI
1801 GAC CGC TTC GAA TTC ATC CCA GTG ACG GCT ACC CTG GAG GCT GAG CCA CCA TAG
601  Asp Arg Phe Glu Phe Ile Pro Val Thr Ala Thr Leu Glu Ala Glu Pro Pro ...
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WHAT IS CLAIMED IS:

1. A plant optimized nucleotide sequence effective for encoding an insecticidal crystal protein (ICP), the nucleotide sequence comprising:

codons effective to encode an ICP with about 589 to about 619 amino acids,

the plant optimized nucleotide sequence being about 71% homologous with a native *Bacillus thuringiensis* nucleotide sequence encoding ICP, and about 63% homologous to a maize nucleotide sequences,

wherein codon usage in the plant optimized nucleotide sequence has a deviation from that of a host plant cell of about 0.23 to about 3.48.

2. A plant optimized nucleotide sequence according to claim 1, wherein codon usage in the plant optimized nucleotide sequence has a deviation from that of a host plant cell of about 1.075.

3. A plant optimized nucleotide sequence according to claim 1, wherein the plant optimized nucleotide sequence is SEQ. ID. NO. 1.

4. A synthetic genetic construct capable of expression in plant cells comprising in sequence from 5' to 3':

a promoter sequence effective to initiate transcription in plant cells;

a translational enhancer sequence;

a plant optimized nucleotide sequence coding for an ICP with about 589 to about 619 amino acids, the plant optimized nucleotide sequence being about 71% homologous with a native *Bacillus thuringiensis* nucleotide sequence encoding ICP, and about 63% homologous to a maize nucleotide sequences, and wherein codon usage in the plant optimized nucleotide sequence

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has a deviation from that of a host plant cell of about 0.23 to about 3.48, and

a polyadenylation sequence,

wherein said promoter sequence, translational enhancer sequence, plant optimized nucleotide sequence and polyadenylation sequence are operably linked.

5. A synthetic gene construct according to claim 4, wherein codon usage in the plant optimized nucleotide sequence has a deviation from that of a host plant cell of about 1.075.

6. A synthetic gene construct according to claim 4, wherein the promoter is selected from the group consisting of inducible promoters, constitutive promoters, temporal-regulated promoters, developmentally-regulated promoters, tissue-preferred, and tissue-specific promoters.

7. A synthetic genetic construct according to claim 4, wherein the promoter is CaMV 35S.

8. A synthetic genetic construct according to claim 4, wherein the translational enhancer is a maize intron.

9. A synthetic genetic construct according to claim 4, wherein the synthetic gene construct is SEQ. ID. NO. 1.

10. A synthetic genetic construct according to claim 8, wherein the maize intron is intron 1 or intron 6 of Adh1.S.

11. A transgenic maize plant wherein cells of the plant have been transformed with a plant optimized nucleotide sequence effective for encoding an insecticidal crystal protein (ICP), where the nucleotide

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sequence comprises codons effective to encode an ICP with about 589 to about 619 amino acids and the plant optimized nucleotide sequence is about 71% homologous to a native *Bacillus thuringiensis* nucleotide sequence encoding ICP and about 63% homologous to maize nucleotide sequences, wherein codon usage in the plant optimized nucleotide sequence has an average deviation from a host plant cell of about 0.23 to about 3.48.

12. A plant optimized nucleotide sequence according to claim 11, wherein codon usage in the plant optimized nucleotide sequence has an average deviation from a host plant cell of about 1.075.

13. A transgenic maize plant according to claim 11, wherein the plant optimized nucleotide sequence is SEQ. ID. NO. 1.

14. A plant seed having in its genome an inheritable synthetic gene, wherein the synthetic gene comprises a plant optimized nucleotide sequence effective for encoding an insecticidal crystal protein (ICP), where the nucleotide sequence comprises codons effective to encode an ICP with about 589 to about 619 amino acids and the plant optimized nucleotide sequence is about 71% homologous to a native *Bacillus thuringiensis* nucleotide sequence encoding ICP and about 63% homologous to maize nucleotide sequences, wherein codon usage in the plant optimized nucleotide sequence has an average deviation from a host plant cell of about 1.075.

15. A plant seed according to claim 14, wherein the plant optimized nucleotide sequence is SEQ. ID. NO. 1.

16. A method of engineering a maize specific optimized insecticidal gene sequence comprising:

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a) determining a percent frequency of nucleic acids in gene coding sequence(s) for a native insecticidal protein;

b) producing a DNA sequence by reverse translating an amino acid coding sequence(s), wherein the resulting reverse translated gene coding sequence(s) contains the same amino acids as a native insecticidal protein, but the nucleic acids coding for the amino acids are replaced with first preferred codon sequences in maize;

c) modifying the DNA sequence produced in step b) by identifying and replacing nucleic acids with second or third preferred codons of maize, where the codons that are replaced eliminate one or more of the following: enzyme restriction sites, exon:intron 5' junctions, poly A addition signals, RNA polymerase termination signals, or TA or GC doublets; and

d) modifying the DNA sequence produced in step c) by identifying G or C sequence blocks that have more than four residues that are the same and substituting G or C with another nucleotide so that the blocks are interrupted with a different nucleotide sequence, such that the protein coded by the sequence is unchanged.

17. The method of claim 16, wherein the native insecticidal protein is produced from *Bacillus thuringiensis*.

18. The method of claim 17, wherein the protein is a toxin produced from *Bacillus thuringiensis* and the toxin is HD73.

19. The method of claim 18, wherein HD73 is HD73 CrylA(c).

20. An ICP gene identified as SEQ. ID. NO. 1.

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21. An ICP gene according to claim 20, wherein the ICP gene is inserted into vector pDAB 917.

22. A maize specific optimized insecticidal gene sequence where the gene sequence contains about 63% first choice codons, between about 22% to about 37% second choice codons, and between about 15% to 0% third and/or fourth choice codons, wherein the total percentage is 100%.

23. A maize specific optimized insecticidal gene sequence according to claim 22, wherein the gene sequence contains about 63% first choice codons, between about 22% to about 37% second choice codons, and between about 15% to 0% third choice codons, wherein the total percentage is 100%.

24. A maize specific optimized insecticidal gene sequence according to claim 22, wherein the gene sequence contains about 63% first choice codons, at least about 22% of second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%.

25. A synthetic gene construct according to claim 4 wherein the promoter and translational enhancer sequence is set forth in Figure 10.

26. A recombinant promoter comprising SEQ. ID. NO. 43.

27. A recombinant promoter comprising SEQ. ID. NO. 46.

28. A transgenic plant comprising a recombinant promoter as described in SEQ. ID. NO. 43 and a plant expressible structural gene placed 3' to the

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promoter, such that the structural gene is expressed under control of the promoter.

29. The transgenic plant of claim 28, wherein the plant is a monocot.

30. The trangenic plant of claim 28 whrerein the plant is a dicot.

31. The transgenic plant of claim 29, wherein the monocot is selected from the group consisting of maize, wheat, sorghum, oats, rye, barley millets, sugar cane, grasses, and rice.

32. The transgenic plant of claim 30, wherein the dicot is selected from the group consisting of soybeans, legume, rapeseed, cotton, sunflower, tomatoes, potatoes, sugar beets, alfalpha, cloves and peanuts.

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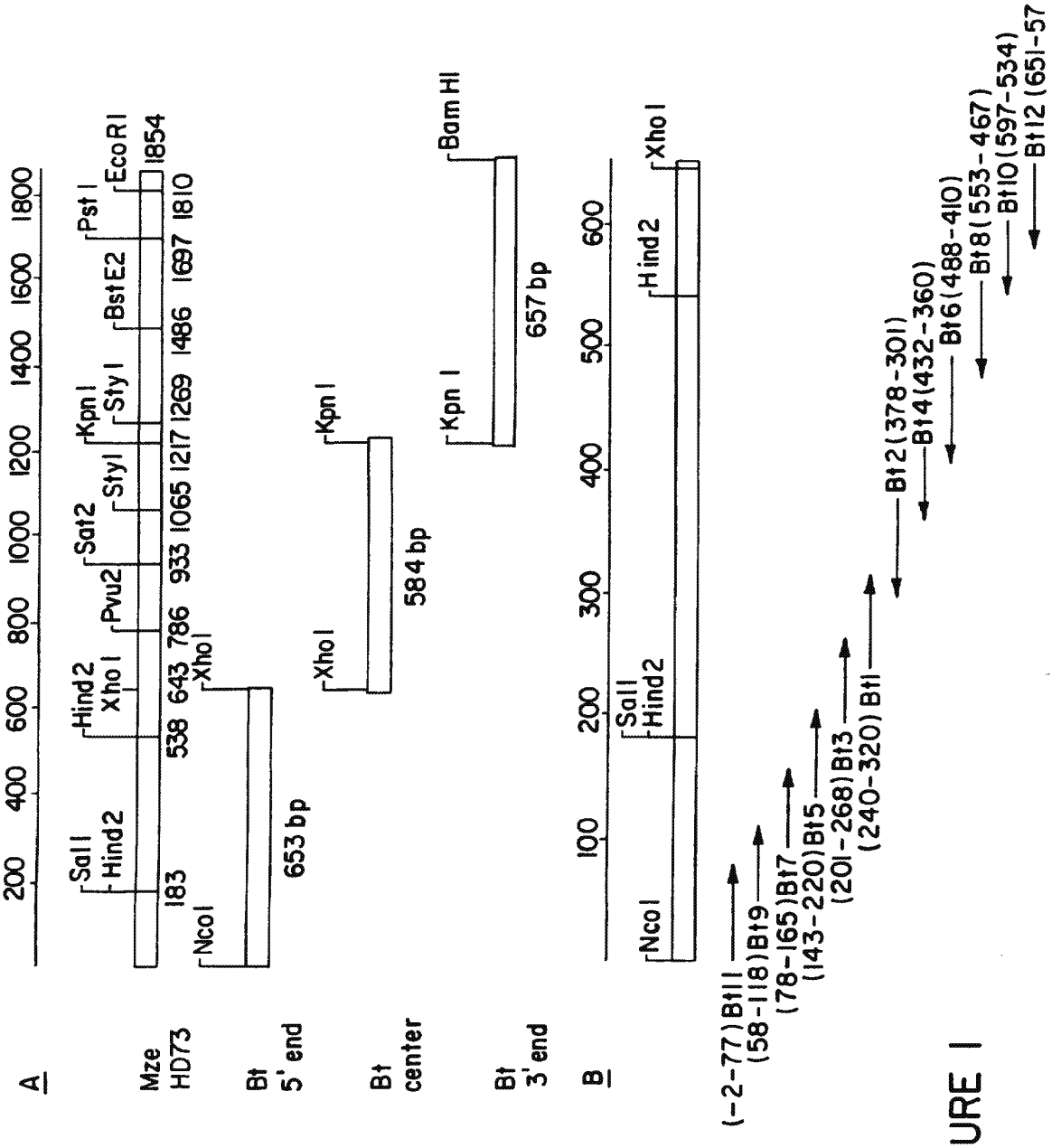


FIGURE 1

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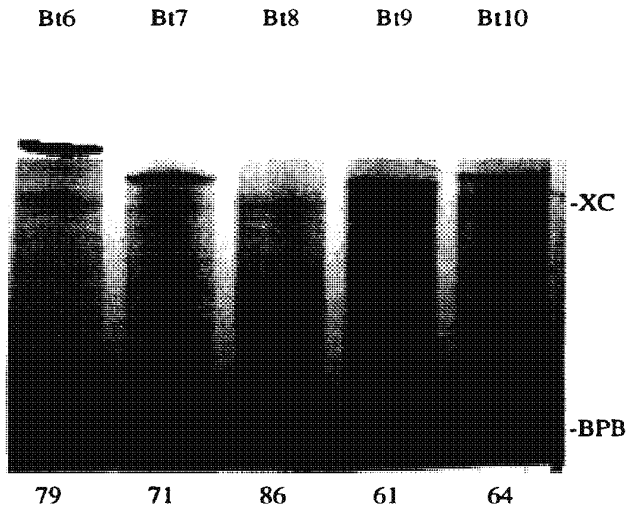


Fig. 2

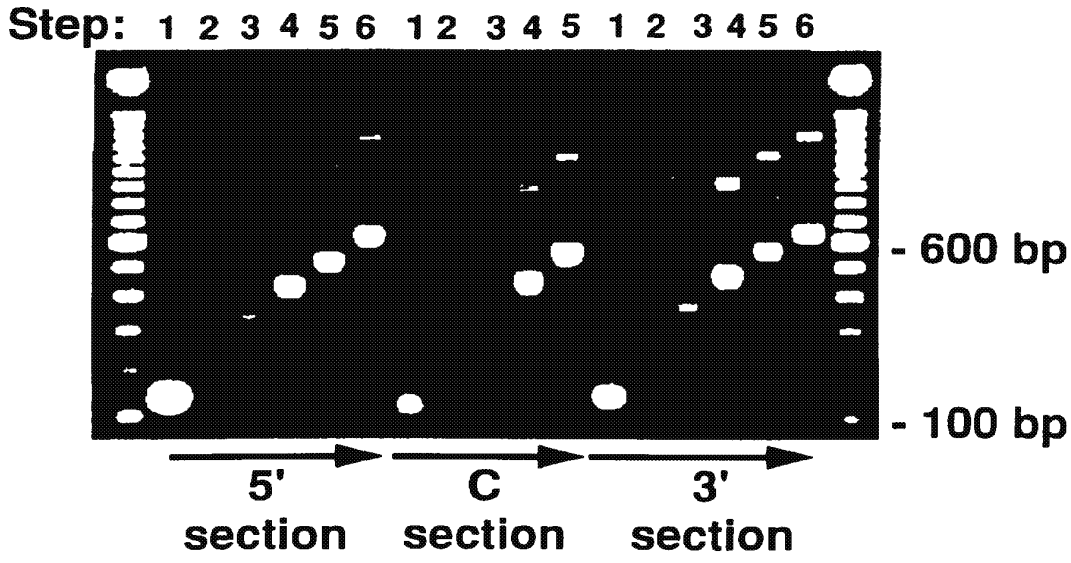


Fig. 3

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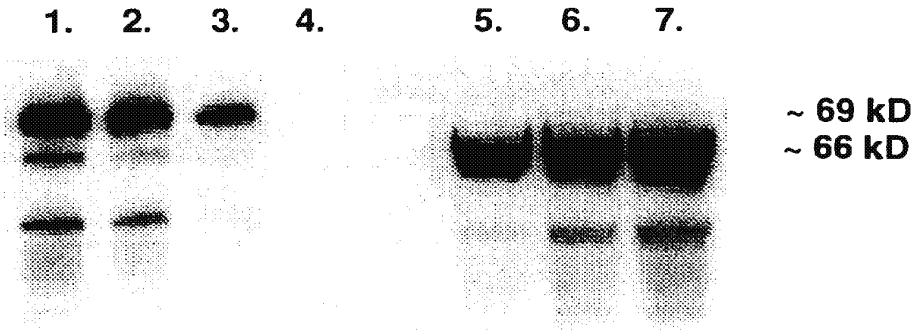
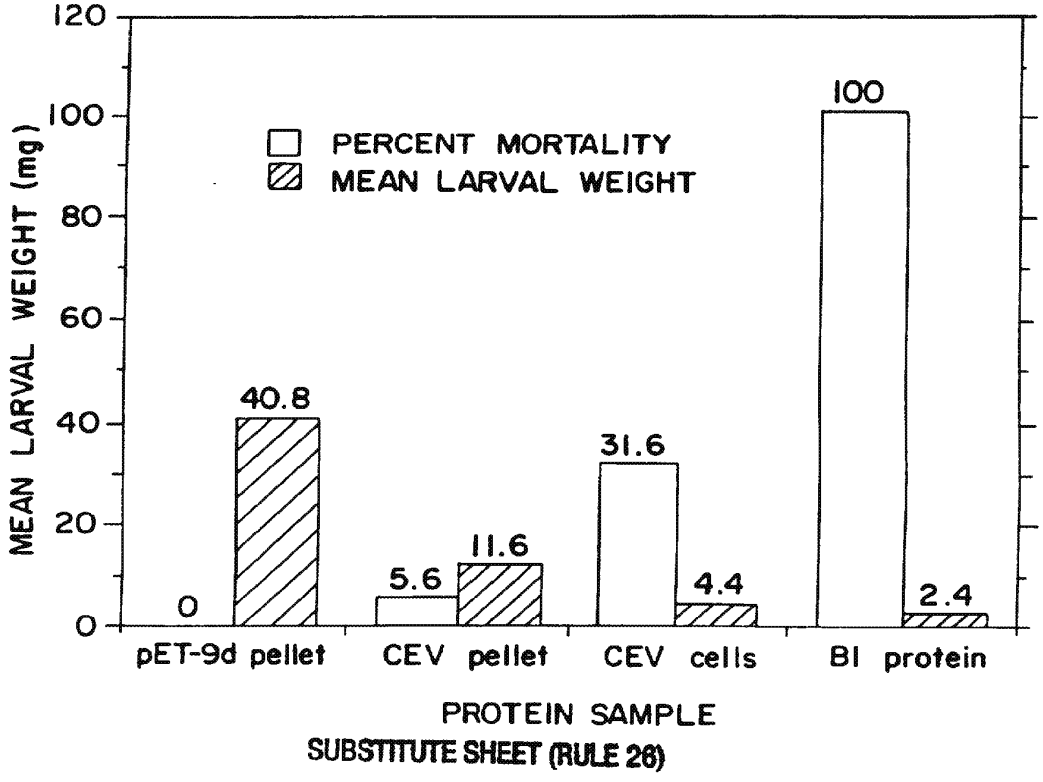


Fig. 4

FIGURE 5



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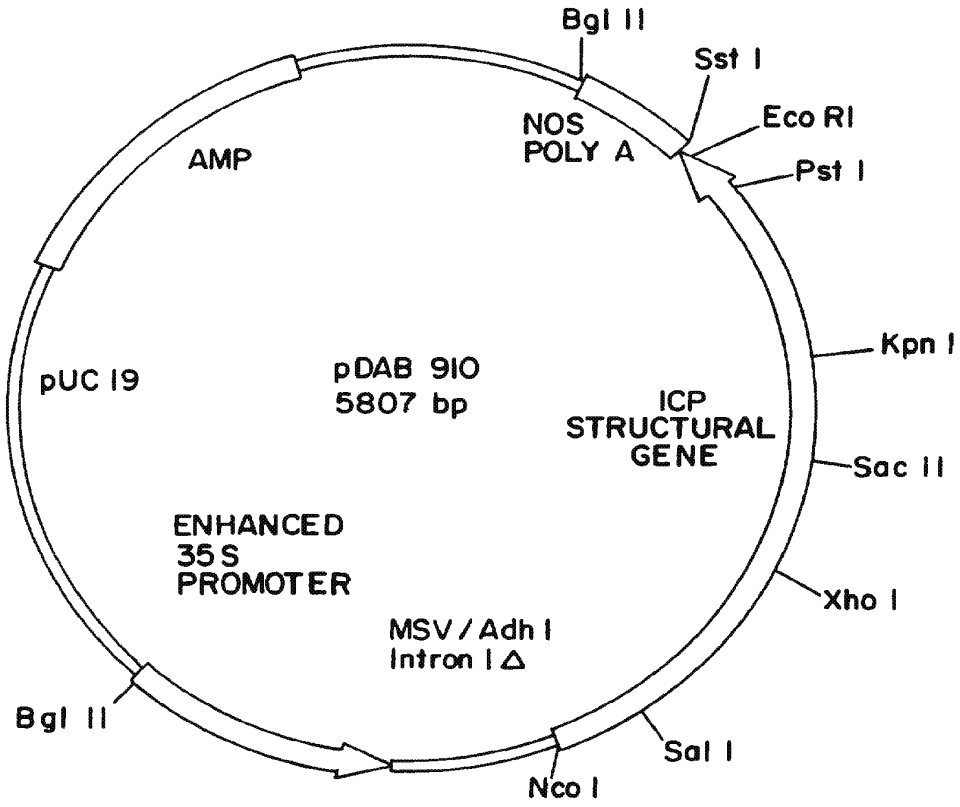


FIGURE 6

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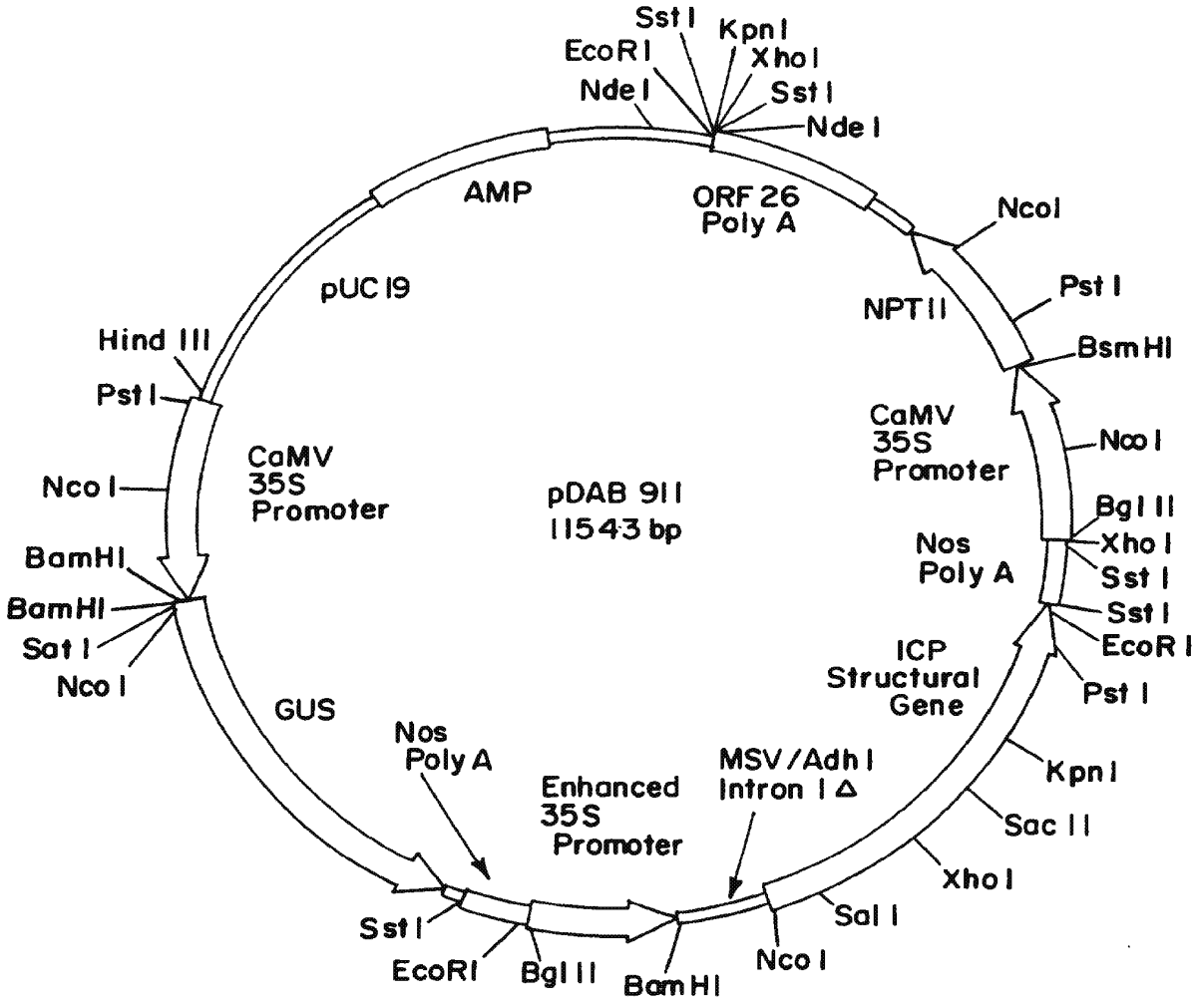
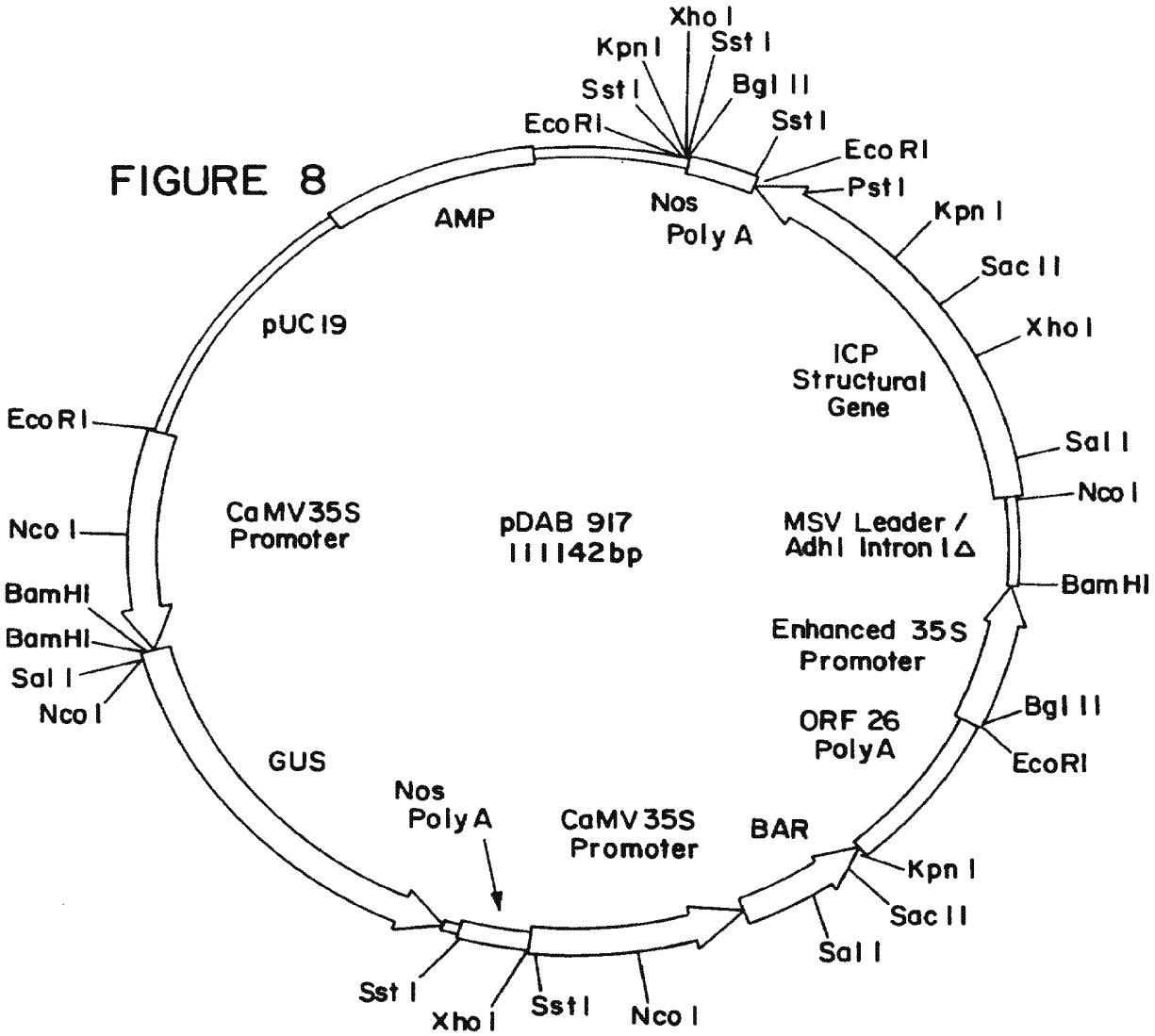


FIGURE 7

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FIGURE 8



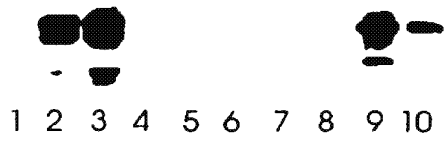


Fig. 9

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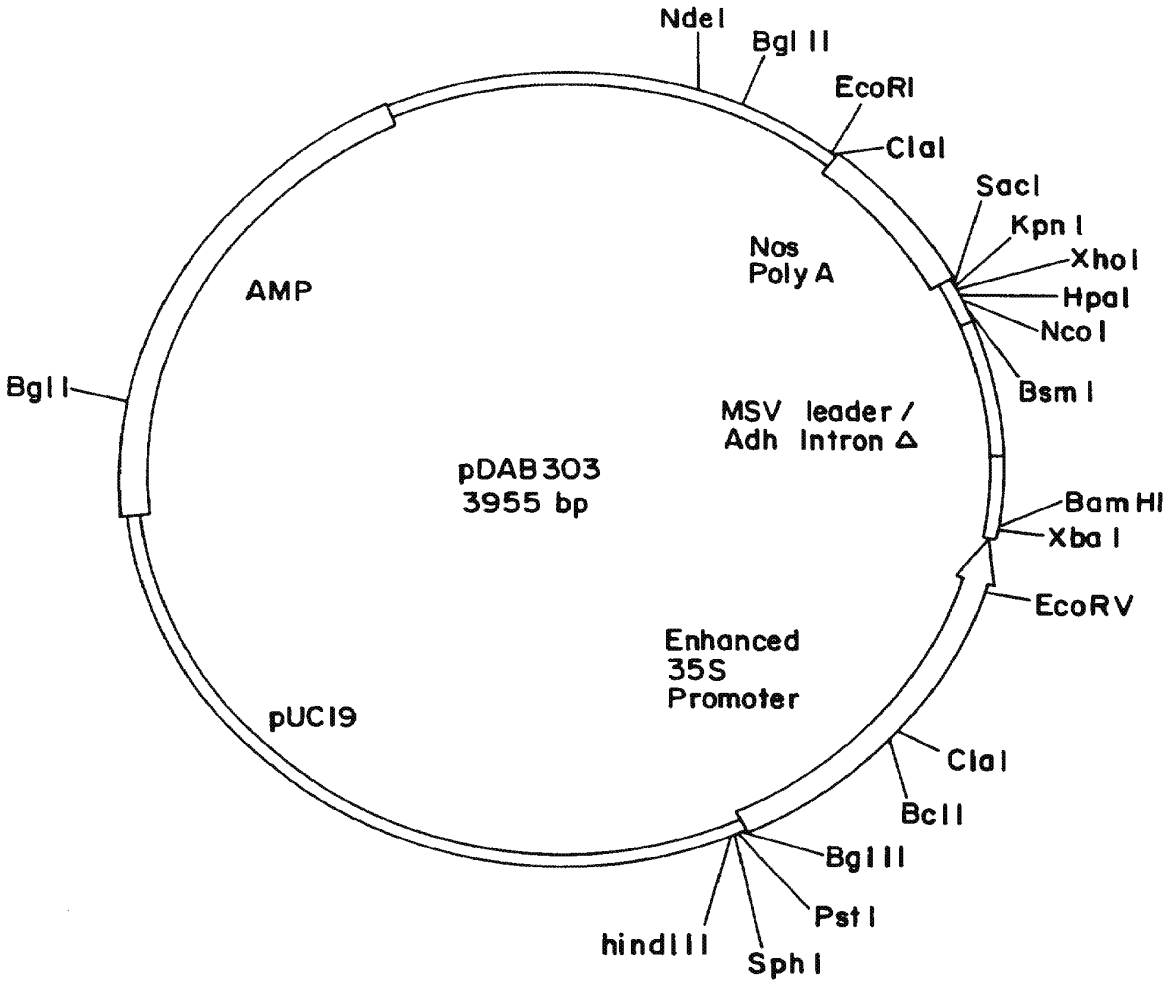


FIGURE 10

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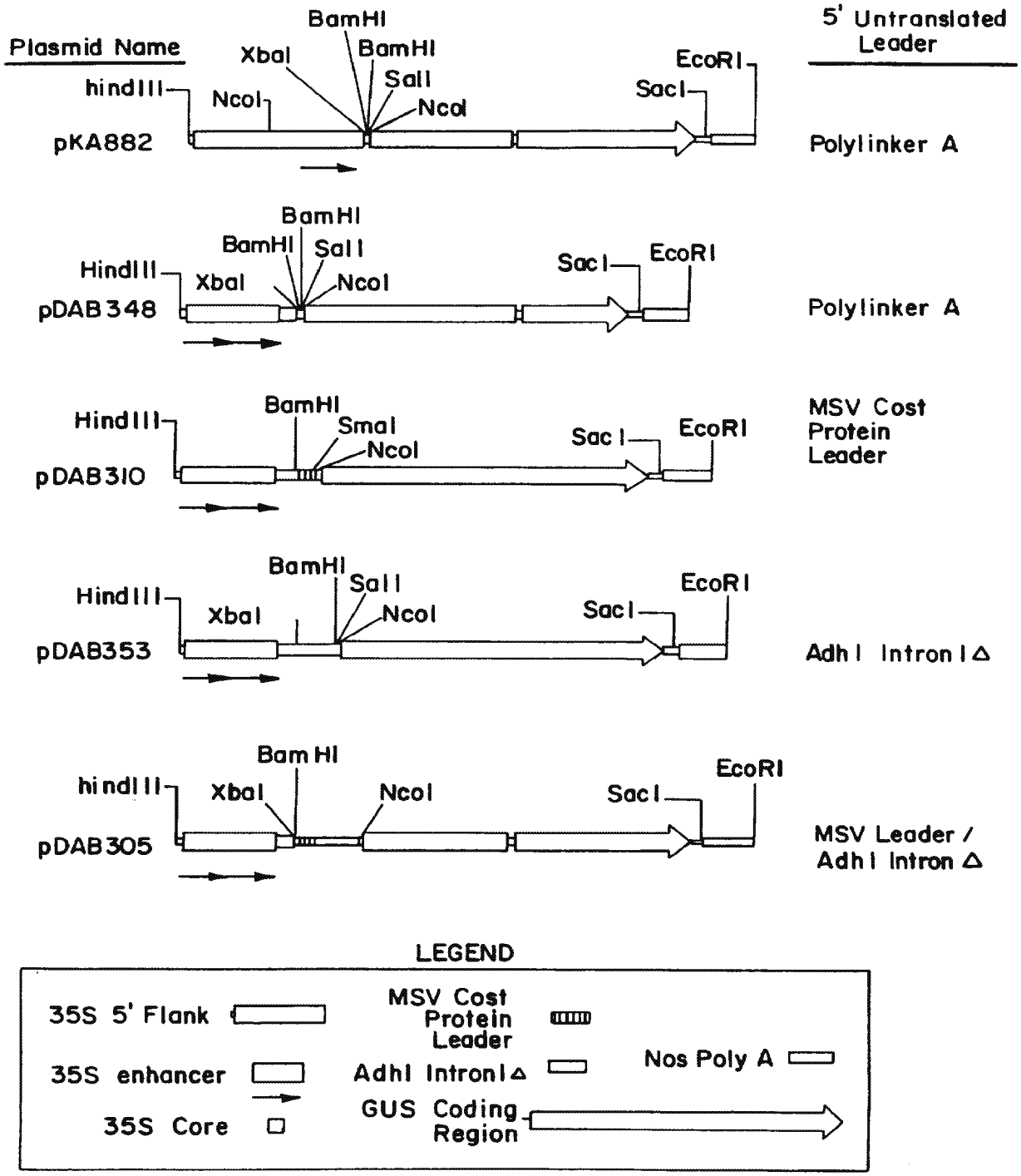


FIGURE 11

SUBSTITUTE SHEET (RULE 26)

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Bayer EX1008 (Part 8 of 9)

INTERNATIONAL SEARCH REPORT

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PCT/US96/16582

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

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Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 359 472 A2 (LUBRIZOL GENETICS INC.) 21 March 1990, pages 5-6, page 9 and page 26.	1-32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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(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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STRUCTURE-BASED DESIGNED HERBICIDE RESISTANT PRODUCTS

Field of the Invention

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.

Background of the Invention

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.

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

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

Brief Description of the Drawings

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 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*.

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 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.

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. 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 *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.

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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 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 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, 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.

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, 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 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 absence and presence of increasing concentrations of imazethapyr (PURSUIT® herbicide) and sulfometuron methyl (OUST® herbicide). The Y axis represents the % activity of the mutant

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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 μM 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

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

Summary of the Invention

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

(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;

(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;

(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

(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;

(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 acetohydroxybutyrate, in the absence and 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-

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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,

(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 activity for viability; and

(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 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;

(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, 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

(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:

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(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;

(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

(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,

(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 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:

(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;

(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;

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(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, 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;

(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-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 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 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 activity for viability; and

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

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

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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,

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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.

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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;

(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.

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

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.

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

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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 Cα 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

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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

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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.,

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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.

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 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 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 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).

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 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. 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

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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.

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 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 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 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 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. 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 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.

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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 Cα 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

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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

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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 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 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 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

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 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 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 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).

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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.

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 μmole 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

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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

(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 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 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 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 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 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 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.

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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.

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,

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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; 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.

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 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 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.

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, 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 promotor 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.

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

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

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

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

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

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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.

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 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 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 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 seed germination and root initiation and proliferation in the presence of herbicide.

Other Applications

The methods and compositions of the present invention 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 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.

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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:

(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;

(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;

(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

(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 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 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 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.

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Exemplification with respect to Preferred Embodiments

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

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 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 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 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 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, 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.

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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 (DH5α) cells transformed with the pGEX-2T vector containing either the maize wild type AHAS gene (vector designation pAC751), the *Arabidopsis* Ser653Asn mutant, or the

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Arabidopsis Ile401Phe mutant were grown overnight in LB broth containing 50 μg/mL ampicillin. The overnight culture of *E. coli* was diluted 1:10 in 1 L LB, 50 μg/mL ampicillin, and 0.1% v/v antifoam A. The culture was incubated at 37°C with shaking 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 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 μg/mL, respectively. Cells were incubated at 30°C for 15 minutes 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 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 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 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 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

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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 μM 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 DH5α /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 eliminated 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

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

Example 4: Enzymatic properties of AHAS variants

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:

A reaction mixture containing 1X AHAS assay buffer (50 mM HEPES pH 7.0, 100 mM pyruvate, 10 mM $MgCl_2$, 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 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 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 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 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% NaN_3 in the presence or absence of FAD and TPP. Furthermore, no degradation products were visible from these stored preparations when resolved in SDS-PAGE gels.

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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

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

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 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 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 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 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 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 activity. This mutant, nonetheless, exhibited a high degree of resistance to PURSUIT® (Figure 10).

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

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 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 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 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) (Figure 11).

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

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 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 mutant AHAS protein in *E. coli* and assessment of its

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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.

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

DNA Constructs:

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 *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 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 *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 *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

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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-Napthyl 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 μM 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

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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:

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 μM PURSUIT® and 100 μM kanamycin. The seedlings that resulted were visually scored for resistance or 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 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, 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*.

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 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 μM CL 299,263 (Figure 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).

All patents, applications, articles, publications, and test methods mentioned above are hereby incorporated by reference.

Many variations of the present invention will suggest 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.

WHAT IS CLAIMED IS

1. A structure-based modelling method for the production of an herbicide resistant AHAS variant protein, said method comprising:

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

(b) modelling one or more herbicides into said three-dimensional structure to localize an 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 mutation alters the affinity of at least one 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, under conditions in which said variant AHAS containing said mutation at said position is produced.

2. A structure-based modelling method as defined in claim 1, further comprising:

(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;

(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 absence and in the presence of said at least one 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 a first herbicide resistant AHAS variant protein is identified having:

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

(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 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 expressed;

wherein said cell requires AHAS activity for viability; and

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

3. A structure-based modelling method as defined in claim 1, wherein said AHAS modelling equivalent is selected from the group consisting of transketolases, carboligases, and pyruvate decarboxylase.

4. A structure-based modelling method as defined in claim 2, wherein said catalytic activity in the absence of said more than one herbicide is more than about 20% of the catalytic activity of said wild-type AHAS in the above of said at least one herbicide.

5. A structure-based modelling method as defined in claim 4, wherein said herbicide is an imidazolinone herbicide and said first herbicide-resistant 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 the presence of imidazolinone herbicides compared to wild type AHAS; and

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

6. A structure-based modelling method as defined in claim 1, wherein said herbicides are selected from the group consisting of imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyl-oxy-benzoic acids, sulfamoylureas sulfocarboxamides, and combinations thereof.

7. A structure-based modelling method as defined in claim 1, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

8. A structure-based modelling method as defined in claim 1, wherein said first cell is *E. coli*.

9. A structure-based modelling method as defined in claim 2, wherein said first and second cells are *E. coli*.

10. A structure-based modelling method as defined in claim 1, wherein said target AHAS protein comprises a protein having the sequence of Figure 1 or a functional equivalent thereof.

11. A structure-based modelling method as defined in claim 1, wherein said mutation is selected from the group consisting of

(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,

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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, and 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, G 583, and 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;

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(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.

12. A structure-based modelling method as defined in claim 11, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing.

13. A structure-based modelling method for the production of herbicide resistant AHAS variant protein, said method comprising:

(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 said target AHAS protein;

(b) modelling one or more herbicides into said three-dimensional structure to localize an 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 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

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

14. A structure-based modelling method as defined in claim 13, further comprising:

(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;

(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 at least one herbicide; and

(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:

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

(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 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 expressed;

wherein said cell requires AHAS activity for viability; and

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

15. A structure-based modelling method as defined in claim 14, wherein said catalytic activity in the absence of said at least one herbicide is more than about 20% of the catalytic activity of said wild-type AHAS.

16. A structure-based modelling method as defined in claim 15, wherein said herbicide is an imidazolinone herbicide and said first herbicide resistant AHAS variant protein has:

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(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 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.

17. A structure-based modelling method as defined in claim 13, wherein said herbicide is selected from the group consisting of imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyl-oxy-benzoic acids, sulfamoylureas, sulfonylcarboxamides, and combinations thereof.

18. A structure-based modelling method as defined in claim 13, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

19. A structure-based modelling method as defined in claim 13, wherein said first cell is *E. coli*.

20. A structure-based modelling method as defined in claim 19, wherein said first and second cells are *E. coli*.

21. A structure-based modelling method as defined in claim 13, wherein said mutation is selected from the group consisting of

(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,

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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;

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(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.

22. A structure-based modelling method as defined in claim 21, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing.

23. A structure-based modelling method for the production of herbicide resistant AHAS variant protein, said method comprising:

(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 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 at least one herbicide for said binding pocket;

(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 produced.

24. A structure-based modelling method as defined in claim 23, 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 acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of said at least one herbicide; and

(h) repeating steps (b)-(g), wherein said DNA 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 at least one herbicide,

(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 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 expressed;

wherein said cell requires AHAS activity for viability; and

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

25. A structure-based modelling method as defined in claim 24, wherein said catalytic activity in the absence of said at least one herbicide is more than about 20% of the catalytic activity of said wild-type AHAS.

26. A structure-based modelling method as defined in claim 25, wherein said herbicide is an imidazolinone herbicide and a first herbicide resistant AHAS variant protein has:

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(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 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.

27. A structure-based modelling method as defined in claim 23, wherein said herbicide is selected from the group consisting of imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyl-oxy-benzoic acids, sulfamoylureas, sulfonylcarboxamides, and combinations thereof.

28. A structure-based modelling method as defined in claim 23, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

29. A structure-based modelling method as defined in claim 23, wherein said cell is *E. coli*.

30. A structure-based modelling method as defined in claim 24, wherein said first and second cells are *E. coli*.

31. A structure-based modelling method as defined in claim 23, wherein said mutation is selected from the group consisting of

(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,

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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;

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(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.

32. A structure-based modelling method as defined in claim 31, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing.

33. An isolated DNA encoding an acetohydroxy acid synthase (AHAS) variant protein, said variant protein 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

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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, or functional equivalents thereof.

34. DNA as defined in claim 33, wherein said modification alters the ability of an herbicide to inhibit the enzymatic activity of said protein.

35. DNA as defined in claim 34, wherein said herbicide is selected from the group consisting of

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imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyl-oxy-benzoic acids, sulfamoylureas, sulfonylcarboxamides, and combinations thereof.

36. DNA as defined in claim 33, wherein said AHAS protein is derived from *Arabidopsis thaliana*.

37. DNA as defined in claim 33, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing.

38. DNA as defined in claim 37, wherein said variant AHAS protein has

(a) in the absence of at least one AHAS inhibiting herbicide,

(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 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 least one herbicide than is wild type AHAS.

39. DNA as defined in claim 33, wherein said variant AHAS has more than about 20% of the catalytic activity of wild-type AHAS.

40. DNA defined in claim 39, wherein said variant AHAS is at least 2-fold more resistant to imidazolinone-based herbicides than to sulfonylurea-based herbicides.

41. A DNA vector comprising the DNA sequence of claim 33 operably linked to a transcription regulatory element.

42. A cell comprising an AHAS encoding DNA sequence derived from a DNA vector as defined in claim 41, wherein said cell is selected from the group consisting of bacterial, fungal, plant, insect, and mammalian cells.

43. A cell as defined in claim 42, comprising a plant cell.

44. A seed comprising a cell as defined in claim 43.

45. A variant AHAS protein comprising a protein encoded by a DNA as defined in claim 33.

46. A variant AHAS protein 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, P314, 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,

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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.

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47. A variant AHAS protein as defined in claim 46, wherein said modification alters the ability of a herbicide to inhibit the enzymatic activity of said protein.

48. A variant AHAS protein as defined in claim 46, wherein said herbicide is selected from the group consisting of an imidazolinones, sulfonylureas, triazolopyrimidine, sulfomamides, pyrimidyl-oxy-benzoic acids, sulfamoylureas, sulfonylcarboxamides, and combinations thereof.

49. A variant AHAS protein as defined in claim 46, wherein said AHAS protein is derived from *Arabidopsis thaliana*.

50. A variant AHAS protein as defined in claim 46, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing or functional equivalents thereof.

51. A variant AHAS protein as defined in claim 46, wherein said variant AHAS protein has

(a) in the absence of said at least one AHAS inhibiting herbicide,

(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 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

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

52. A variant AHAS protein as defined in claim 46, wherein said variant AHAS has more than about 20% of the catalytic activity of wild-type AHAS.

53. A method for conferring herbicide resistance on a cell, said method comprising:

(a) cloning a DNA as defined in claim 33 into a compatible expression vector; and

(b) transforming said DNA into said cell, under conditions wherein said gene is expressed at sufficient levels to confer herbicide resistance on said cell.

54. A cell prepared according to the method of claim 53.

55. A plant comprising a cell as defined in claim 54.

56. A method as defined in claim 53, wherein said mutated gene encodes a different amino acid at least one of positions 53, 128, 135, or combinations thereof.

57. A method as defined in claim 56, wherein said AHAS gene comprises the *Arabidopsis thaliana* AHAS gene.

58. A method as defined in claim 53, wherein said cell is selected from the group consisting of bacterial, fungal, plant, insect, and mammalian cells.

59. A method as defined in claim 58, wherein said cell is a plant cell.

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60. A method as defined in claim 59, wherein said cell is in a seed.

61. A method for production of herbicide-resistant AHAS protein, said method comprising:

(a) selecting an amino acid position in an AHAS protein as a target for mutation;

(b) mutating DNA encoding AHAS to produce a mutated DNA encoding a mutation at said position;

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

(d) expressing wild-type AHAS protein in parallel in a second cell;

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

(f) assaying said wild-type and said variant AHAS proteins, for catalytic activity in conversion of pyruvate to acetolactate, in the absence and in the presence of imidazolinone or sulfonylurea herbicides; and

(g) repeating steps (a)-(g), wherein said mutated DNA is used as the AHAS-encoding DNA in step (b) until an herbicide resistant AHAS protein having:

(i) catalytic activity in the absence of herbicides of more than about 20% of the catalytic activity of said 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 is identified.

62. A method as defined in claim 61, wherein said herbicide is selected from the group consisting of imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyl-oxy-benzoic acids,

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sulfamoylureas, sulfonylcarboxamides, and combinations thereof.

63. A method as defined in claim 61, wherein said AHAS protein is derived from *Arabidopsis thaliana*.

64. A method as defined in claim 61, wherein said cell is *E. coli*.

65. A method as defined in claim 61, wherein said target AHAS protein comprises a protein having the sequence of Figure 1.

66. A method as defined in claim 65, wherein said mutation is selected from the group consisting of

(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,

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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.

67. A method as defined in claim 66, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing.

68. A method for controlling weeds in a crop, said method comprising cultivating a crop comprising herbicide resistant plants as defined in claim 55, and treating

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said crop with a weed controlling effective amount of said herbicide.

69. A method for controlling weeds in a crop, said method comprising cultivating a crop comprising herbicide resistant plants as defined in claim 55, and treating said crop with a weed controlling effective amount of an herbicidal composition comprising said herbicide.

70. A structure-based modelling method for the preparation of a first herbicide which inhibits AHAS activity, said method comprising:

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

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

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

71. A structure-based modelling method as defined in claim 70, wherein said AHAS modelling functional equivalent is selected from the group consisting of transketolases, carboligases, and pyruvate decarboxylase.

72. A structure-based modelling method as defined in claim 70, wherein said first herbicide contains at least one functional group that interacts with a functional group of said binding pocket.

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73. A structure-based modelling method as defined in claim 70, wherein said first herbicide binds to at least an AHAS activity-inhibiting effective portion of said binding pocket.

74. A structure-based modelling method as defined in claim 70, wherein said first herbicide inhibits said catalytic activity of said AHAS to less than about 20% of the catalytic activity of wild-type AHAS.

75. A structure-based modelling method as defined in claim 70, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

76. A structure-based modelling method as defined in claim 70, wherein said target AHAS protein comprises a protein having the sequence of Figure 1 or a functional equivalent thereof.

77. A structure-based modelling method for the production of a first herbicide which inhibits AHAS activity, said method comprising:

(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 said target AHAS protein;

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

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

78. A structure-based modelling method as defined in claim 77, wherein said first herbicide contains at least one functional group that interacts with a functional group of said binding pocket.

79. A structure-based modelling method as defined in claim 77, wherein said first herbicide binds to at least an AHAS activity inhibiting effective portion of said binding pocket.

80. A structure-based modelling method as defined in claim 77, wherein said first herbicide inhibits said catalytic activity of said AHAS to less than about 20% of the catalytic activity of wild-type AHAS.

81. A structure-based modelling method as defined in claim 77, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

82. A structure-based modelling method for the production of a first herbicide which inhibits AHAS activity, said method comprising:

(a) designing a non-peptide herbicide which will interact with an AHAS activity inhibiting-effective portion of a binding pocket of an AHAS protein, wherein said herbicide inhibits said AHAS activity sufficiently to destroy the viability of a cell which requires AHAS activity for viability.

83. A cell transformed with a DNA as defined in claim 33, wherein said DNA is expressed in said cell at sufficient levels to confer herbicide resistance on said cell.

84. A plant transformed with a DNA as defined in claim 33, wherein said DNA is expressed in said plant at sufficient levels to confer herbicide resistance on said plant.

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FIG. Ia

A

*1 * GSAASPAMP*10 *MAPPATPLRP*20 *WGPTDPRKGA*

*60 *TRSPVIANHL*70 *FRHEQGEAFA*00 *ASGYARSSGR*

Arg128 Phe135

*120*AITGQVPRRM*130*IGTDAFQETP*140*IVEVTRSITK*

*180*LVDIPKDIQQ*190*QMAVPVWDKP*200*MSLPGYIARL*

*240*ARSGEELRRF*250*VELTGIPVTT*260*TLMQLGNFPS*

*300*GVRFDDRVTG*310*KIEAFASRAK*320*IVHVDIDPAE*

*360*SKKSFDFGSW*370*NDELDQQKRE*380*FPLGYKTSNE*

*420*WAAQYYTYKR*430*PRQWLSSAGL*440*GAMGFGLPAA*

*480*IRIENLPVKV*490*FVLNNQHLGM*500*VVQWEDRFYK*

*540*PAVRVTKKNE*550*VRAAIKKMLE*560*TPGPYLLDII*

A

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Met 53

A

30 *DILVESLERC*40 *GVRDVFAYPG*50 *GASMEIHQAL

90 *VGVCIATSGP*100*GATNLVSALA*110*DALLDSVPMV

150*HNYLVLDVDD*160*IPRVVQEAFF*170*LASSGRPGPV

210*PKPPATELLE*220*QVLRLVGESR*230*RPVLYVGGGC

270*DDPLSLRMLG*280*MHGTVYANYA*290*VDKADLLLAL

330*IGKNKQPHVS*340*ICADVKLALQ*350*GMNALLEGST

390*EIQPQYAIQV*400*LDELTKGEAI*410*IGTGVGQHQM

450*AGASVANPGV*460*TVVDIDGDGS*470*FLMNVQELAM

510*ANRAHTYLGN*520*PENESEIYPD*530*FVTIAKGFNI

570*VPHQEHVLPM*500*IPSGGAFKDM*590*ILDGDGRTVY

A

FIG. 1b

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FIG. 2a

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POX          *   *          *   *          *24 *   TNILAGA*31 *AVIKVLEAWG*41 *VDHLYGIPGG*51 *SINSIMDALS
                                                 |||||||     |||||||| ||     ||||||||||     || |||||||
AHAS_pred    *1  *GSAASPAMPM*11 *APPATPLRPW*21 *GPTDPRKGAD*31 *ILVESLERCG*41 *VRDVFAYPGG*51 *ASMEIHQALT

POX          *61 *AERDRIHYIQ*71 *VRHEEVGAMA*81 *AAADAKLTGK*91 *IGVCFGSAGP*101*GGTHLMNGLY*111*DAREDHVPVL
                  | |||||||     ||||||||||     ||| ||||||     ||||||||||     ||||||||||     ||| ||||||
AHAS_pred    *61 *RS PVIANHL*70 *FRHEQGEAFA*80 *ASGYARSSGR*90 *VGVCIATSGP*100*GATNLVSALA*110*DALLDSVPMV

POX          *121*ALIGQFGTTG*131*MNMDTFQEMN*141*ENPIYADVAD*151*YNVTAVNAAT*161*LPHVIDEAIR*171*RAYAHQ GVA
                  |||||||||      ||||||||||     | ||| ||||     ||| ||||||     |||||||||      |||||| |||
AHAS_pred    *120*AITGQVPRRM*130*IGTDAFQETP*140*IVEVTRSITK*150*HNYLVLDVDD*160*IPRVVQEAFF*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*LEQVLRLVGE*228*SRRPVLYVGG

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

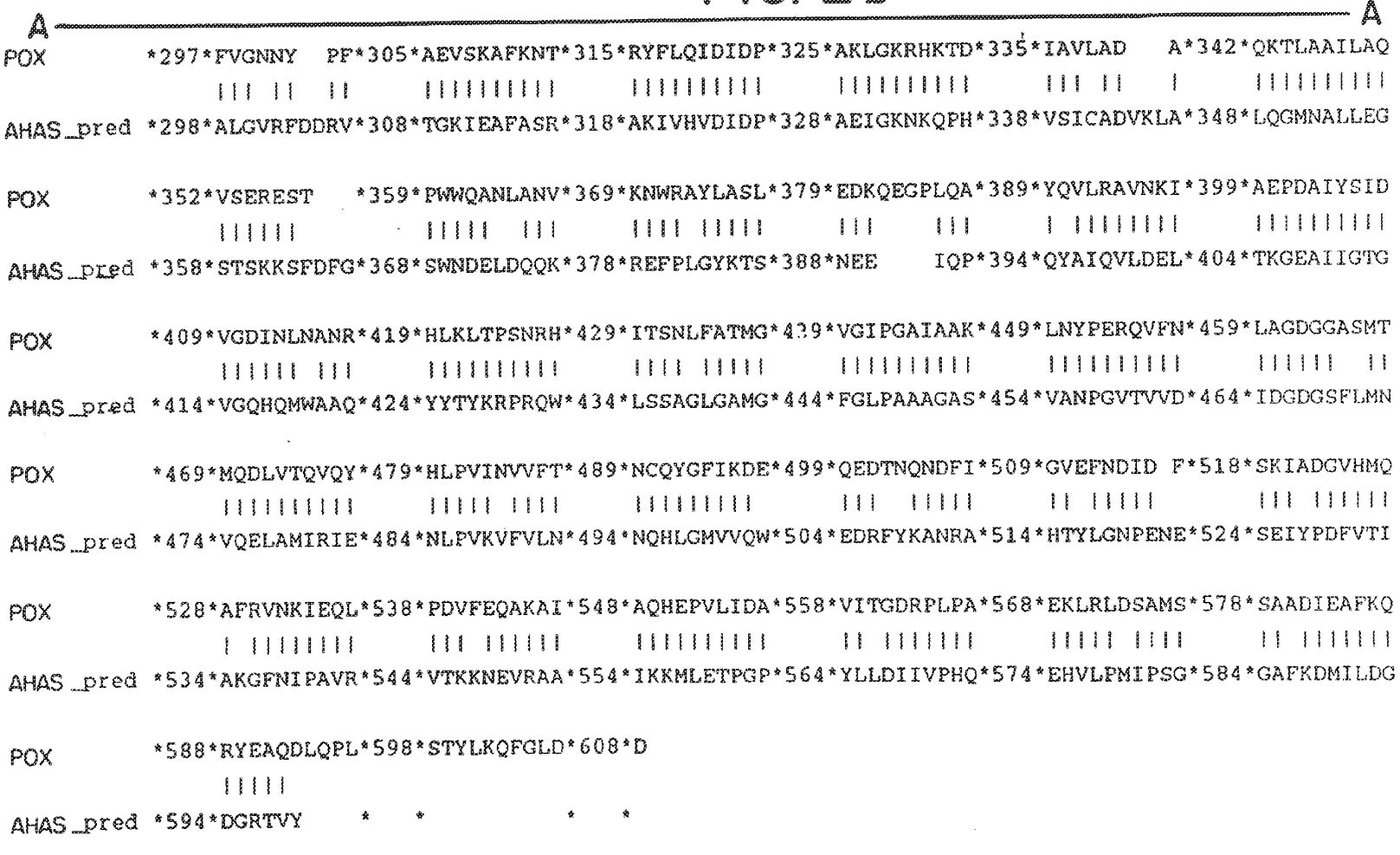
A ——————————————————————————————————————————————————————————————————————————————————————————— A
```

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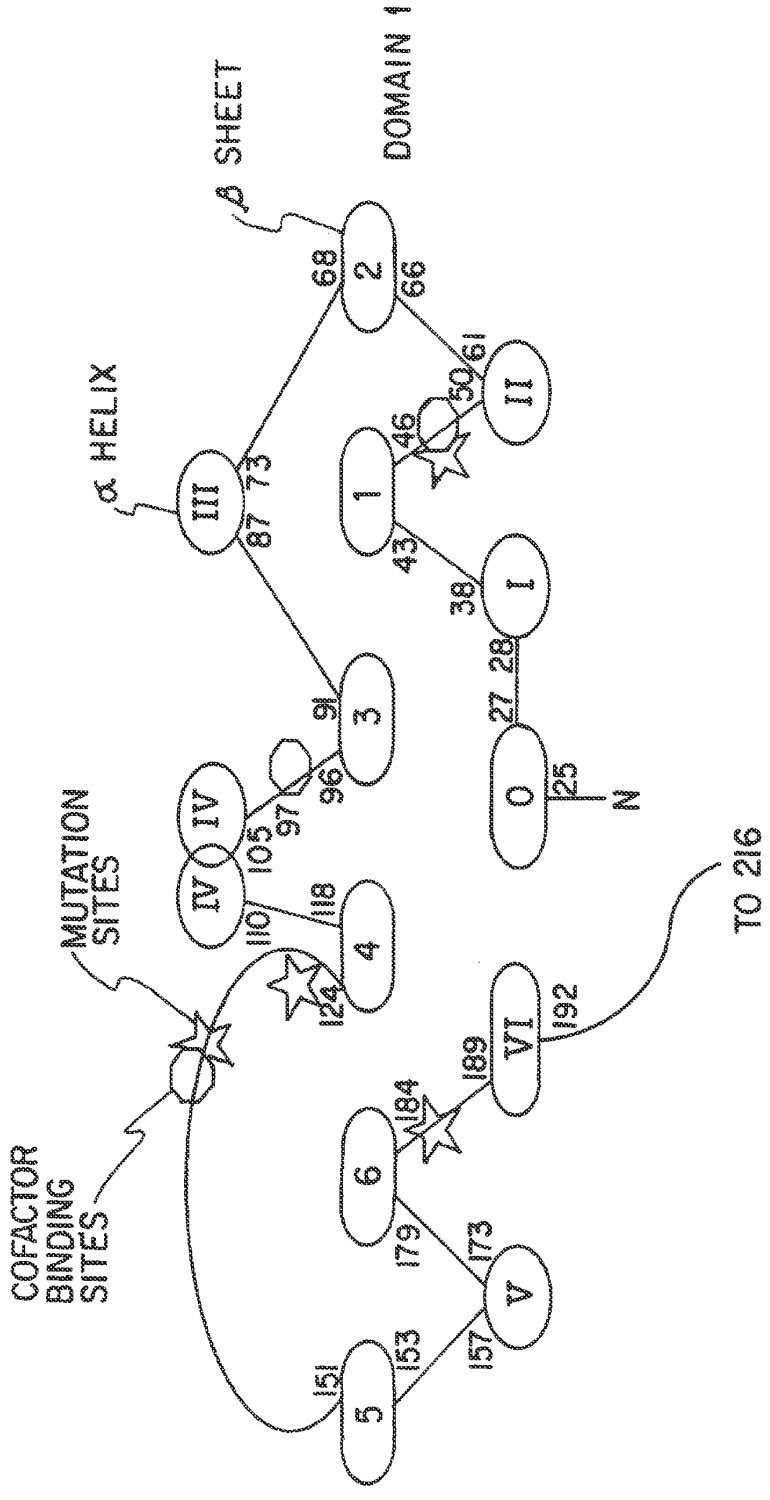
FIG. 2b



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FIG. 3a



COR-AAD1-00004257

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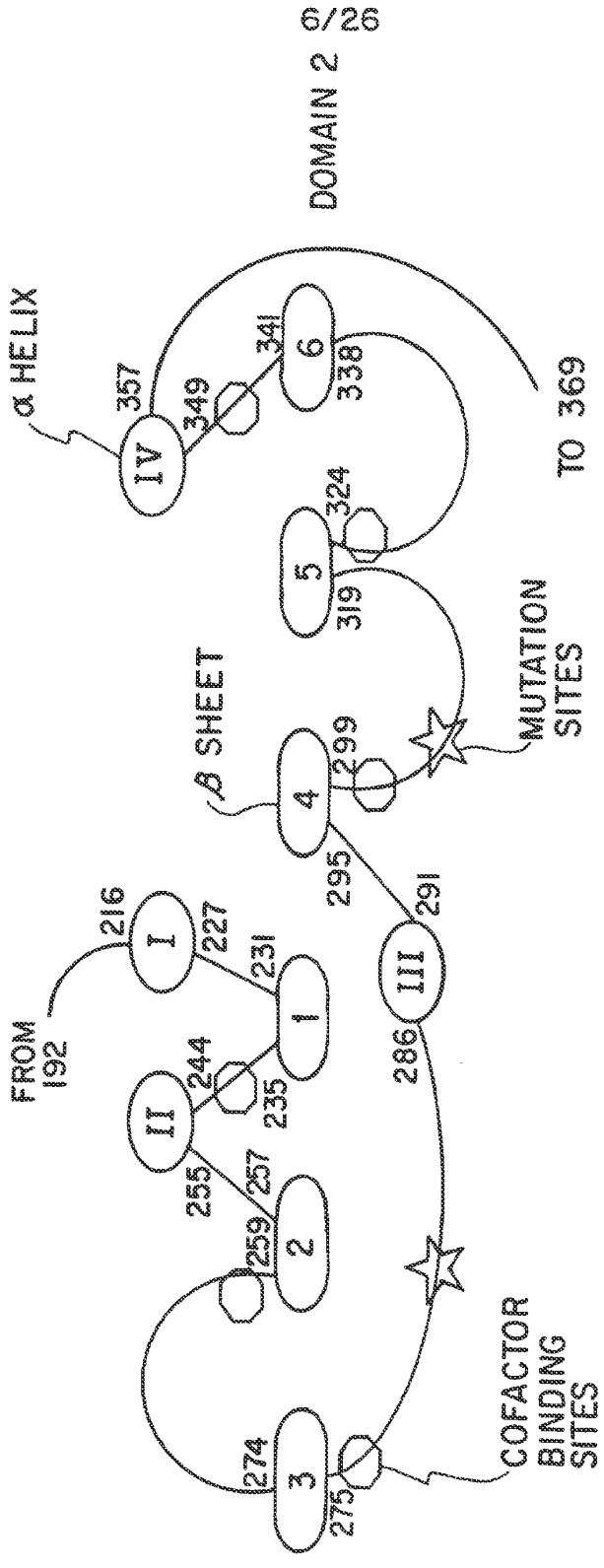


FIG. 3b

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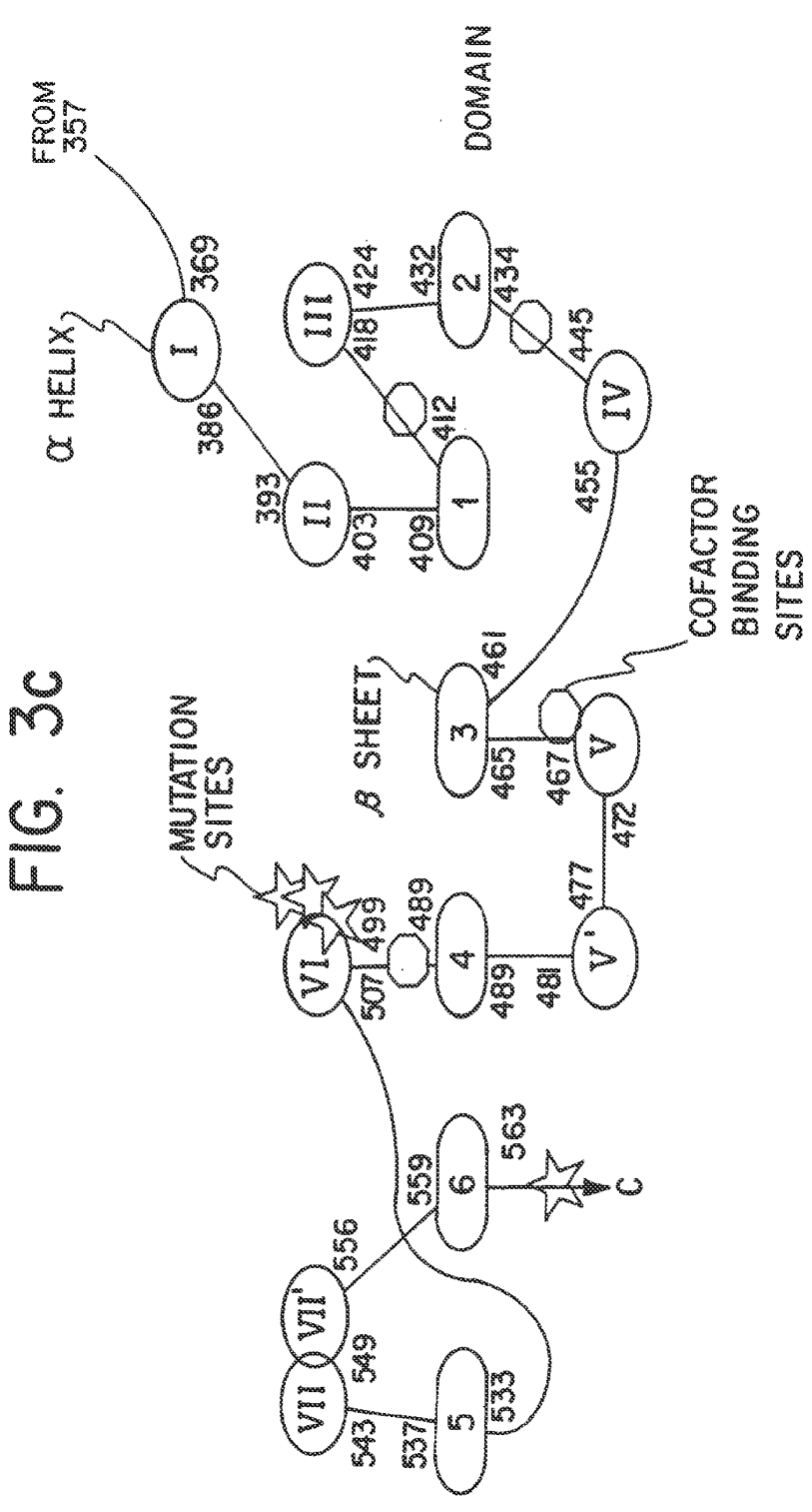
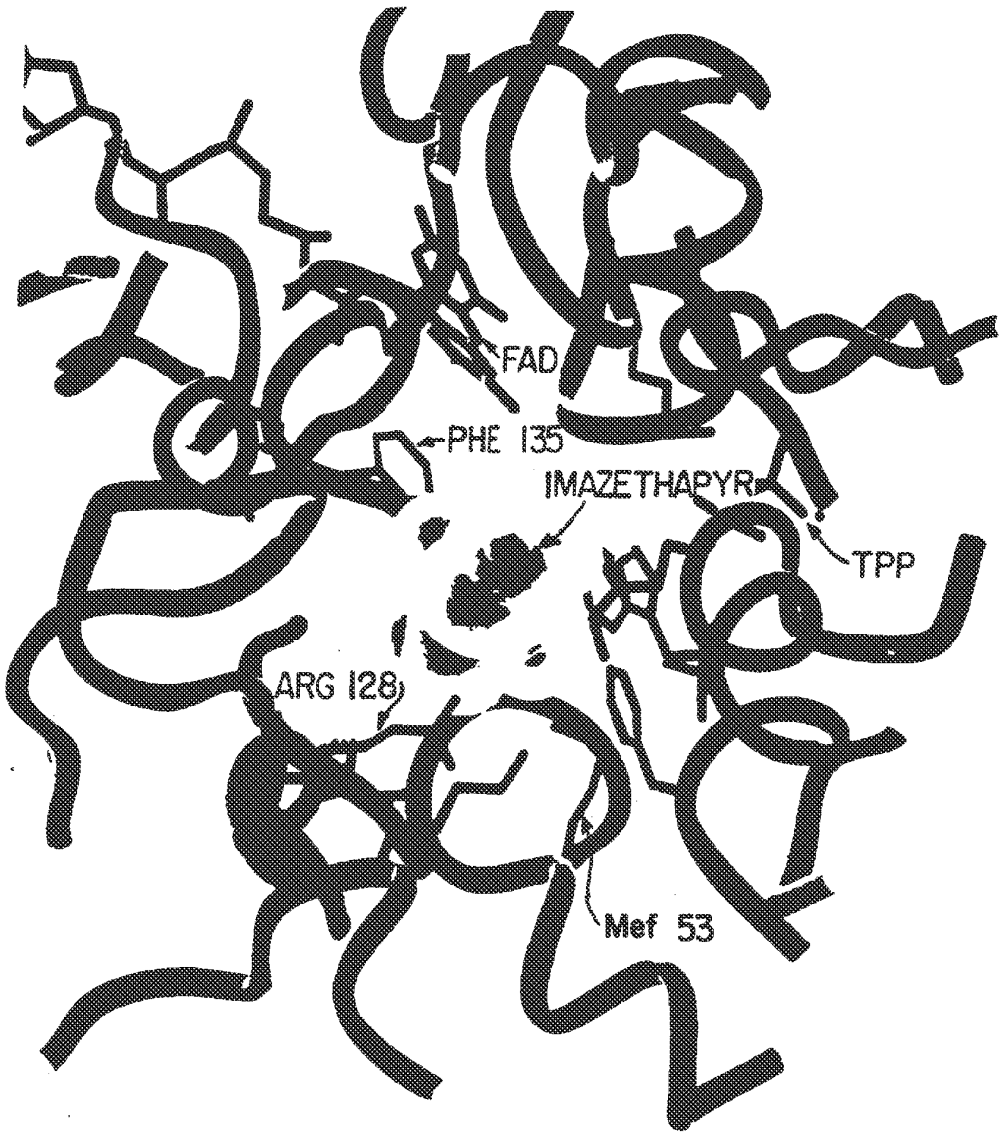


FIG. 3c

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FIG. 4



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FIG. 5a

```
           1                                                        50
Pac751     .......... .......... .......... .......... ..........
Maizeals2  .......... .......... .......... ..MATAAAAS TALTGATTAA
Maizeals1  .......... .......... .......... ..MATAATAA AALTGATTAT
Tobac1     MAAA...APS PSSSAFSKTL SPSSSTSSTL LPRSTFPFPH HPHKTTPPPL
Tobac2     MAAA...AAA PSPS.FSKTL SSSSSKSSTL LPRSTFPFPH HPHKTTPPPL
Athcsr12   MAAATTTTTT SSSISFSTKP SPSSSKSPLP ISRFSLPFSL NPNKSSSSSR
Bnaal3     MAAA....TS SSPISLTAKP ...SSKSPLP ISRFSLPFSL TPQKPSSRLH
Bnaal2     .........M ASFSFFGTIP S.....SPTK ASVFSLPVSV TTLPSFPRRR
Consensus  MAAA---ATS -S-SSFS--P SPSSSKSPT- -SRFTLPFS- TPLK--P---

           51                                                       100
Pac751     .......... .......... .GSAASPAMP MAPPATPLRP WGPTDPRKGA
Maizeals2  PKARRRAHLL ATRRALAAPI RCSAASPAMP MAPPATPLRP WGPTDPRKGA
Maizeals1  PKSRRRAHHL ATRRALAAPI RCSALSRATP TAPPATPLRP WGPNEPRKGS
Tobac1     HLTHTHIHIH SQRRRFTISN VISTNQKVSQ TEKTETFVSR FAPDEPRKGS
Tobac2     HLTPT..HIH SQRRRFTISN VISTTQKVSE TQKAETFVSR FAPDEPRKGS
Athcsr12   RRGIKSSSPS SISAVLNTTT NVTTTPSPTK PTKPETFISR FAPDQPRKGA
Bnaal3     R.......PL AISAVLNSPV NV....APEK TDKIKTFISR YAPDEPRKGA
Bnaal2     .........AT RVSVSANSKK DQDRTAS..R RENPSTFSSK YAPNVPRSGA
Consensus  --TR-RAH-L -IRR-LN-PI --S-TS-A-P T-KP-TF-SR -APDEPRKGA

A ————————————————————————————————————————————————————————————— A
```


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FIG. 5b

A

```
           101                                                        150
Pac751     DILVESLERC GVRDVFAYPG GASMEIHQAL TRSPVIANHL FRHEQGEAFA
Maizeals2  DILVESLERC GVRDVFAYPG GASMEIHQAL TRSPVIANHL FRHEQGEAFA
Maizeals1  DILVEALERC GVRDVFAYPG GASMEIHQAL TRSPVIANHL FRHEQGEAFA
Tobac1     DVLVEALERE GVTDVFAYPG GASMEIHQAL TRSSIIRNVL PRHEQGGVFA
Tobac2     DVLVEALERE GVTDVFAYPG GASMEIHQAL TRSSIIRNVL PRHEQGGVFA
Athcsr12   DILVEALERQ GVETVFAYPG GASMEIHQAL TRSSSIRNVL PRHEQGGVFA
Bnaal3     DILVEALERQ GVETVFAYPG GASMEIHQAL TRSSTIRNVL PRHEQGGVFA
Bnaal2     DILVEALERQ GVDVVFAYPG GASMEIHQAL TRSNTIRNVL PRHEQGGIFA
Consensus  DILVEALER- GV-DVFAYPG GASMEIHQAL TRSSVIRNVL PRHEQGGVFA
```

EQUIVALENT TO MAIZE MET 53

```
           151                                                        200
Pac751     ASGYARSSGR VGVCIATSGP GATNLVSALA DALLDSVPMV AITGQVPRRM
Maizeals2  ASGYARSSGR VGVCIATSGP GATNLVSALA DALLDSVPMV AITGQVPRRM
Maizeals1  ASAYARSSGR VGVCIATSGP GATNLVSALA DALLDSVPMV AITGQVPRRM
Tobac1     AEGYARATGF PGVCIATSGP GATNLVSGLA DALLDSVPIV AITGQVPRRM
Tobac2     AEGYARATGF PGVCIATSGP GATNLVSGLA DALLDSVPIV AITGQVPRRM
Athcsr12   AEGYARSSGK PGICIATSGP GATNLVSGLA DALLDSVPLV AITGQVPRRM
Bnaal3     AEGYARSSGK PGICIATSGP GATNLVSGLA DAMLDSVPLV AITGQVPRRM
Bnaal2     AEGYARSSGK PGICIATSGP GAMNLVSGLA DALFDSVPLI AITGQVPRRM
Consensus  AEGYARSSG- PGVCIATSGP GATNLVSGLA DALLDSVP-V AITGQVPRRM
```

EQUIVALENT TO MAIZE ARG 128

B

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FIG. 5c

B ——— B

EQUIVALENT TO MAIZE PHE 135

```
            201                                                  250
Pac751      IGTDAFQETP IVEVTRSITK HNYLVLDVDD IPRVVQEAFF LASSGRPGPV
Maizeals2   IGTDAFQETP IVEVTRSITK HNYLVLDVDD IPRVVQEAFF LASSGRPGPV
Maizeals1   IGTDAFQETP IVEVTRSITK HNYLVLDVDD IPRVVQEAFF LASSGRPGPV
Tobac1      IGTDAFQETP IVEVTRSITK HNYLVMDVED IPRVVREAFF LARSGRPGPI
Tobac2      IGTDAFQETP IVEVTRSITK HNYLVMDVED IPRVVREAFF LARSGRPGPV
Athcsr12    IGTDAFQETP IVEVTRSITK HNYLVMDVED IPRIIEEAFF LATSGRPGPV
Bnaal3      IGTDAFQETP IVEVTRSITK HNYLVMDVDD IPRIVQEAFF LATSGRPGPV
Bnaal2      IGTMAFQETP VVEVTRTITK HNYLVMEVDD IPRIVREAFF LATSVRPGPV
Consensus   IGTDAFQETP IVEVTRSITK HNYLVMDVDD IPRVVQEAFF LA-SGRPGPV

            251                                                  300
Pac751      LVDIPKDIQQ QMAVPVWDKP MSLPGYIARL PKPPATELLE QVLRLVGESR
Maizeals2   LVDIPKDIQQ QMAVPVWDKP MSLPGYIARL PKPPATELLE QVLRLVGESR
Maizeals1   LVDIPKDIQQ QMAVPAWDTP MSLPGYIARL PKPPATEFLE QVLRLVGESR
Tobac1      LIDVPKDIQQ QLVIPDWDQP MRLPGYMSRL PKLPNEMLLE QIVRLISESK
Tobac2      LIDVPKDIQQ QLVIPDWDQP MRLPGYMSRL PKLPNEMLLE QIVRLISESK
Athcsr12    LVDVPKDIQQ QLAIPNWEQA MRLPGYMSRM PKPPEDSHLE QIVRLISESK
Bnaal3      LVDVPKDIQQ QLAIPNWDQP MRLPGYMSRL PQPPEVSQLG QIVRLISESK
Bnaal2      LIDVPKDVQQ QFAIPNWEQP MRLPLYMSTM PKPPKVSHLE QILRLVSESK
Consensus   LVDVPKDIQQ QLAIPNWDQP MRLPGYMSRL PKPPA--LLE QI-RL-SESK
```

C ——— C

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FIG. 5d

C ——— C

	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	LNSSDELGRF	VELTGIPVAS	TLMGLGSYPC	DD.ELSLHML
Bnaal3	RPVLYVGGGS	LNSSEELGRF	VELTGIPVAS	TLMGLGSYPC	ND.ELSLQML
Bnaal2	RPVLYVGGGC	LNSSEELRRF	VELTGIPVAS	TFMGLGSYPC	DDEEFSLQML
Consensus	RPVLYVGGGC	-NSSEELRRF	VELTGIPVAS	TLMGLG-FP-	DD-ELSLRML

	351				400
Pac751	GMHGTVYANY	AVDKADLLLA	LGVRFDDRVT	GKIEAFASRA	KIVHVDIDPA
Maizeals2	GMHGTVYANY	AVDKADLLLA	LGVRFDDRVT	GKIEAFASRA	KIVHVDIDPA
Maizeals1	GMHGTVYANY	AVDKADLLLA	FGVRFDDRVT	GKIEAFAGRA	KIVHIDIDPA
Tobac1	GMHGTVYANY	AVDSSDLLLA	FGVRFDDRVT	GKLEAFASRA	KIVHIDIDSA
Tobac2	GMHGTVYANY	AVDSSDLLLA	FGVRFDDRVT	GKLEAFASRA	KIVHIDIDSA
Athcsr12	GMHGTVYANY	AVEHSDLLLA	FGVRFDDRVT	GKLEAFASRA	KIVHIDIDSA
Bnaal3	GMHGTVYANY	AVEHSDLLLA	FGVRFDDRVT	GKLEAFASRA	KIVHIDIDSA
Bnaal2	GMHGTVYANY	AVEYSDLLLA	FGVRFDDRVT	GKLEAFASRA	KIVHIDIDST
Consensus	GMHGTVYANY	AVDKSDLLLA	FGVRFDDRVT	GKLEAFASRA	KIVHIDIDSA

D ——— D

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FIG. 5e

D

```
          401                                                  450
Pac751    EIGKNKQPHV SICADVKLAL QGMNALLEGS TSKKSFDFGS WNDELDQQKR
Maizeals2 EIGKNKQPHV SICADVKLAL QGMNALLEGS TSKKSFDFGS WNDELDQQKR
Maizeals1 EIGKNKQPHV SICADVKLAL QGMNTLLEGS TSKKSFDFGS WHDELDQQKR
Tobac1    EIGKNKQPHV SICADIKLAL QGLNSILESK EGKLKLDFSA WRQELTEQKV
Tobac2    EIGKNKQPHV SICADIKLAL QGLNSILESK EGKLKLDFSA WRQELTVQKV
Athcsr12  EIGKNKTPHV SVCGDVKLAL QGMNKVLENR AEELKLDFGV WRNELNVQKQ
Bnaal3    EIGKNKTPHV SVCGDVKLAL QGMNKVLENR AEELKLDFGV WRSELSEQKQ
Bnaal2    EIGKNKTPHV SVCCDVQLAL QGMNEVLENR RD..VLDFGE WRCELNEQRL
Consensus EIGKNKQPHV SICADVKLAL QGMN-VLE-- T-KLKLDFGS 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 VLDELTNGNA IISTGVGQHQ MWAAQYYKYR
Tobac2    KYPLNFKTFG DAIPPQYAIQ VLDELTNGSA IISTGVGQHQ MWAAQYYKYR
Athcsr12  KFPLSFKTFG EAIPPQYAIK VLDELTDGKA IISTGVGQHQ MWAAQFYNYK
Bnaal3    KFPLSFKTFG EAIPPQYAIQ VLDELTQGKA IISTGVGQHQ MWAAQFYKYR
Bnaal2    KFPLRYKTFG EEIPPQYAIQ LLDELTDGKA IITTGVGQHQ MWAAQFYRFK
Consensus KFPLG-KTFG E-IPPQYAIQ VLDELTKG-A IISTGVGQHQ MWAAQYY-YK
```

E

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FIG. 5f

E

```
          501                                                        550
Pac751    RPRQWLSSAG LGAMGFGLPA AAGASVANPG VTVVDIDGDG SFLMNVQELA
Maizeals2 RPRQWLSSAG LGAMGFGLPA AAGASVANPG VTVVDIDGDG SFLMNVQELA
Maizeals1 RPRQWLSSAG LGAMGFGLPA AAGAAVANPG VTVVDIDGDG SFLMNIQELA
Tobac1    KPRQWLTSGG LGAMGFGLPA AIGAAVGRPD EVVVDIDGDG SFIMNVQELA
Tobac2    KPRQWLTSGG LGAMGFGLPA AIGAAVGRPD EVVVDIDGDG SFIMNVQELA
Athcsr12  KPRQWLSSGG LGAMGFGLPA AIGASVANPD AIVVDIDGDG SFIMNVQELA
Bnaal3    KPRQWLSSSG LGAMGFGLPA AIGASVANPD AIVVDIDGDG SFIMNVQELA
Bnaal2    KPRQWLSSGG LGAMGFGLPA AMGAAIANPG AVVVDIDGDG SFIMNIQELA
Consensus KPRQWLSSGG 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 NPSNEAEIFP
Tobac2    TIKVENLPVK IMLLNNQHLG MVVQWEDRFY KANRAHTYLG NPSNEAEIFP
Athcsr12  TIRVENLPVK VLLLNNQHLG MVMQWEDRFY KANRAHTFLG DPAQEDEIFP
Bnaal3    TIRVENLPVK ILLLNNQHLG MVMQWEDRFY KANRAHTYLG DPARENEIFP
Bnaal2    TIRVENLPVK VLLINNQHLG MVLQWEDHFY AANRADSFLG DPANPEAVFP
Consensus TIRVENLPVK V-LLNNQHLG MVVQWEDRFY KANRAHTYLG NP-NESEIFP
```

F

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FIG. 5g

```
F                                                                                F
            601                                                             650
Pac751      DFVTIAKGFN IPAVRVTKKN EVRAAIKKML ETPGPYLLDI IVPHQEHVLP
Maizeals2   DFVTIAKGFN IPAVRVTKKN EVRAAIKKML ETPGPYLLDI IVPHQEHVLP
Maizeals1   DFVAIAKGFN IPAVRVTKKS EVHAAIKKML EAPGPYLLDI IVPHQEHVLP
Tobac1      NMLKFAEACG VPAARVTHRD DLRAAIQKML DTPGPYLLDV IVPHQEHVLP
Tobac2      NMLKFAEACG VPAARVTHRD DLRAAIQKML DTPGPYLLDV IVPHQEHVLP
Athcsr12    NMLLFAAACG IPAARVTKKA DLREAIQTML DTPGPYLLDV ICPHQEHVLP
Bnaal3      NMLQFAGACG IPAARVTKKE ELREAIQTML DTPGPYLLDV ICPHQEHVLP
Bnaal2      DMLLFAASCG IPAARVTRRE DLREAIQTML DTPGPFLLDV VCPHQDHVLP
Consensus   -ML-FAKACG IPAARVTKK- -LRAAIQKML DTPGPYLLDV IVPHQEHVLP

            651                                                             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                                                                                G
```


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FIG. 5h

G——G

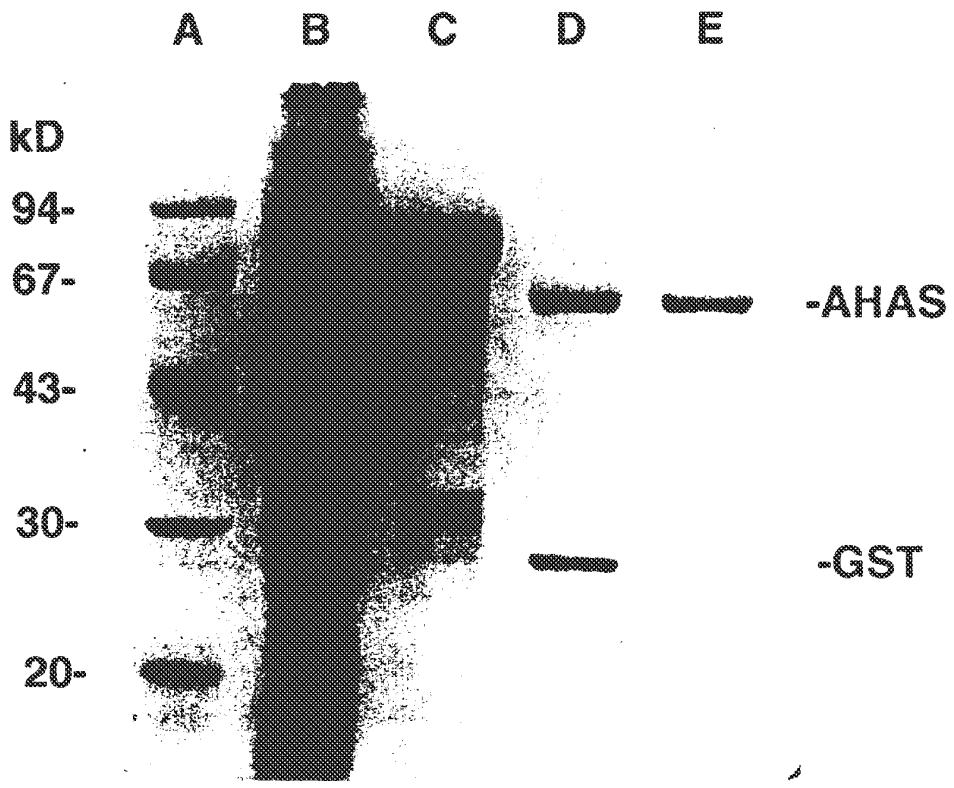
Pac751 - maize als2 AHAS isozyme as expressed from the pAC751 E. coli
expression vector (same as figure 1)
Maizeals2 - maize als2 AHAS isozyme (plant)
Maizeals1 - maize als1 AHAS isozyme (plant)
Tobac1 - tobacco AHAS SuRA isozyme (plant)
Tobac2 - tobacco AHAS SuRB isozyme (plant)
Athcsr12 - Arabidopsis 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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COR-AAD1-00004268

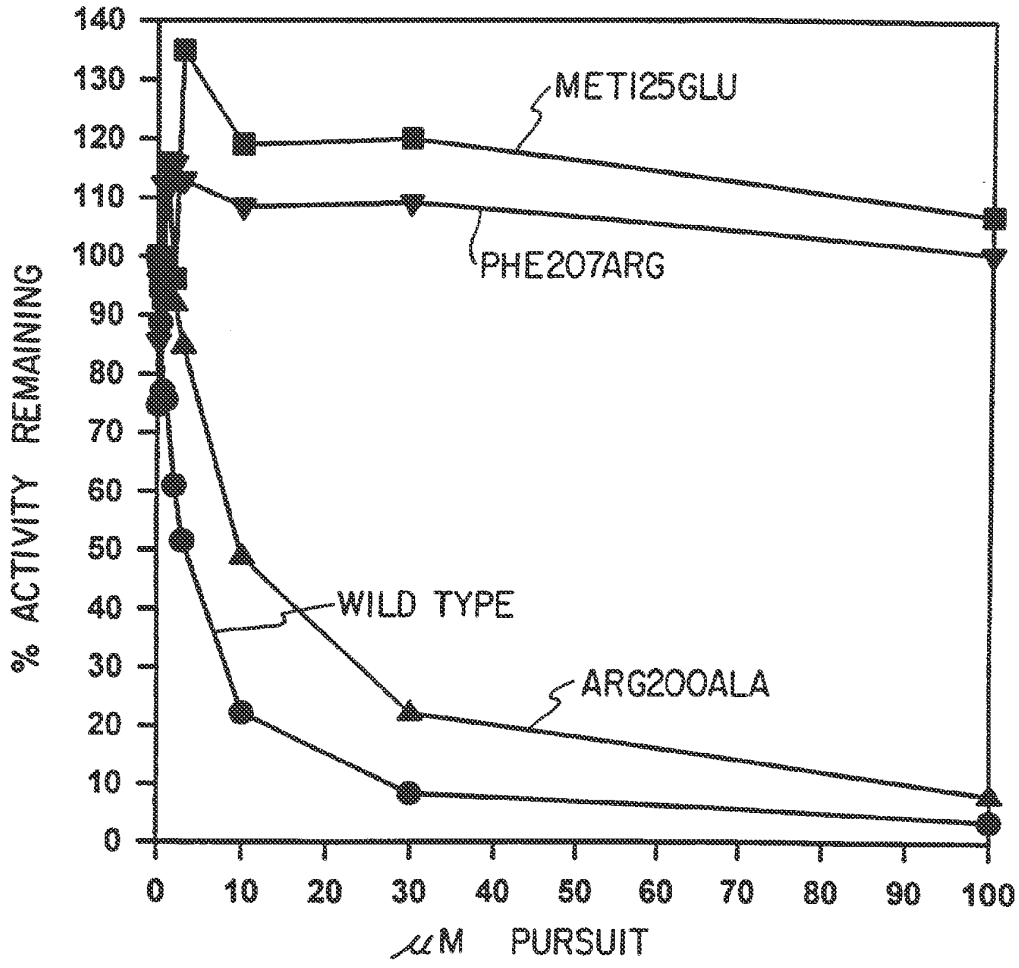
Bayer EX1008 (Part 8 of 9)

FIG. 6



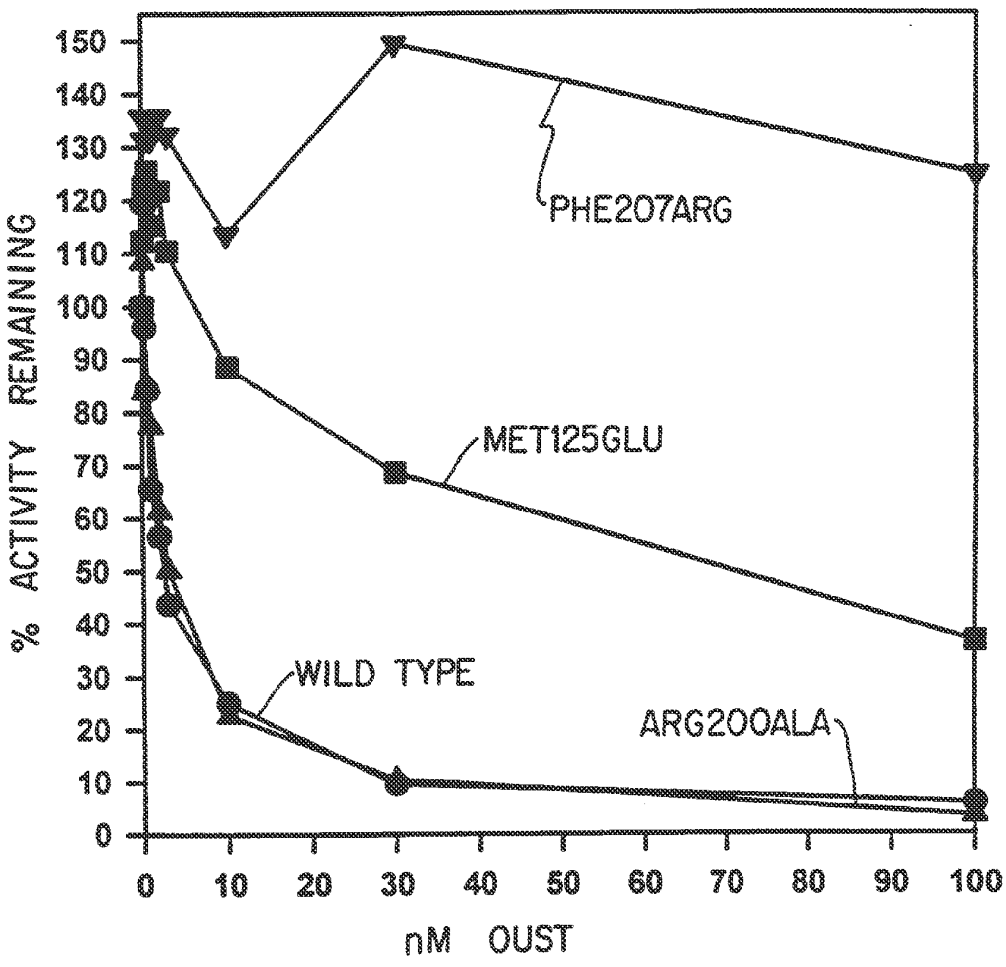
COR-AAD1-00004269
Bayer EX1008 (Part 8 of 9)

FIG. 7



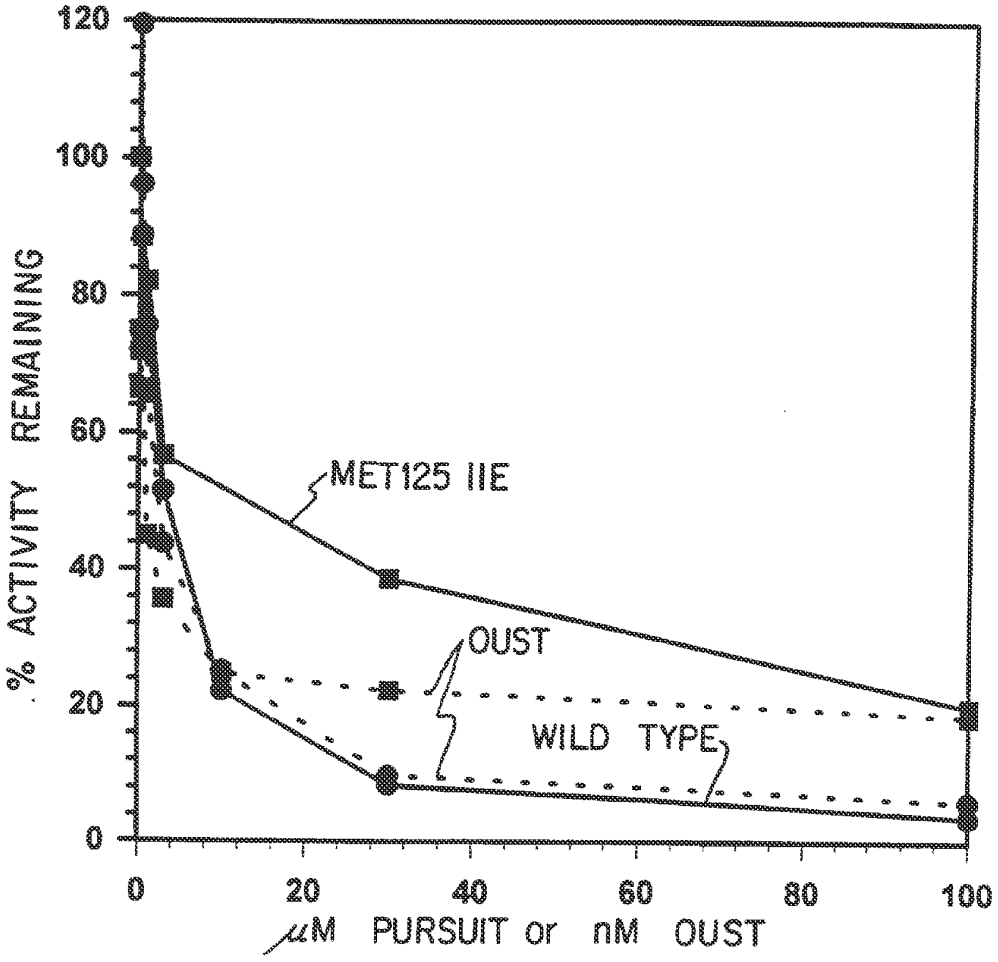
COR-AAD1-00004270
Bayer EX1008 (Part 8 of 9)

FIG. 8



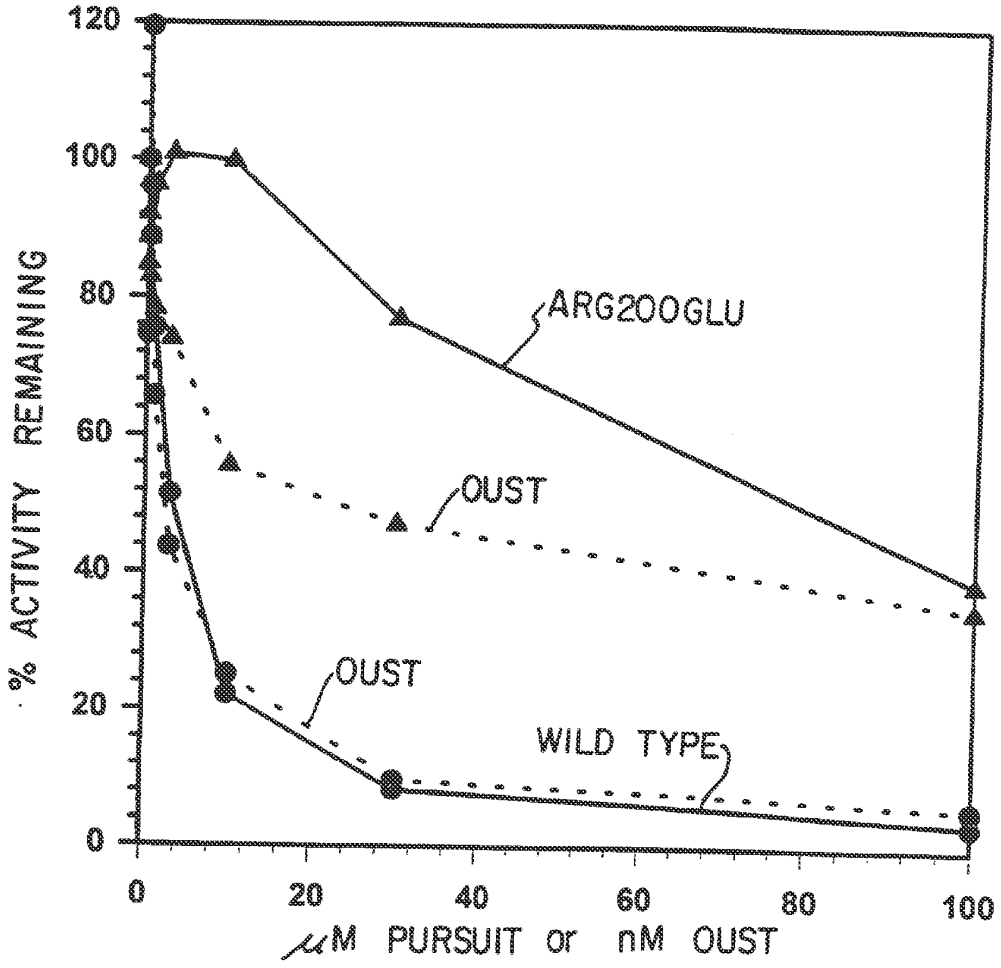
COR-AAD1-00004271
Bayer EX1008 (Part 8 of 9)

FIG. 9



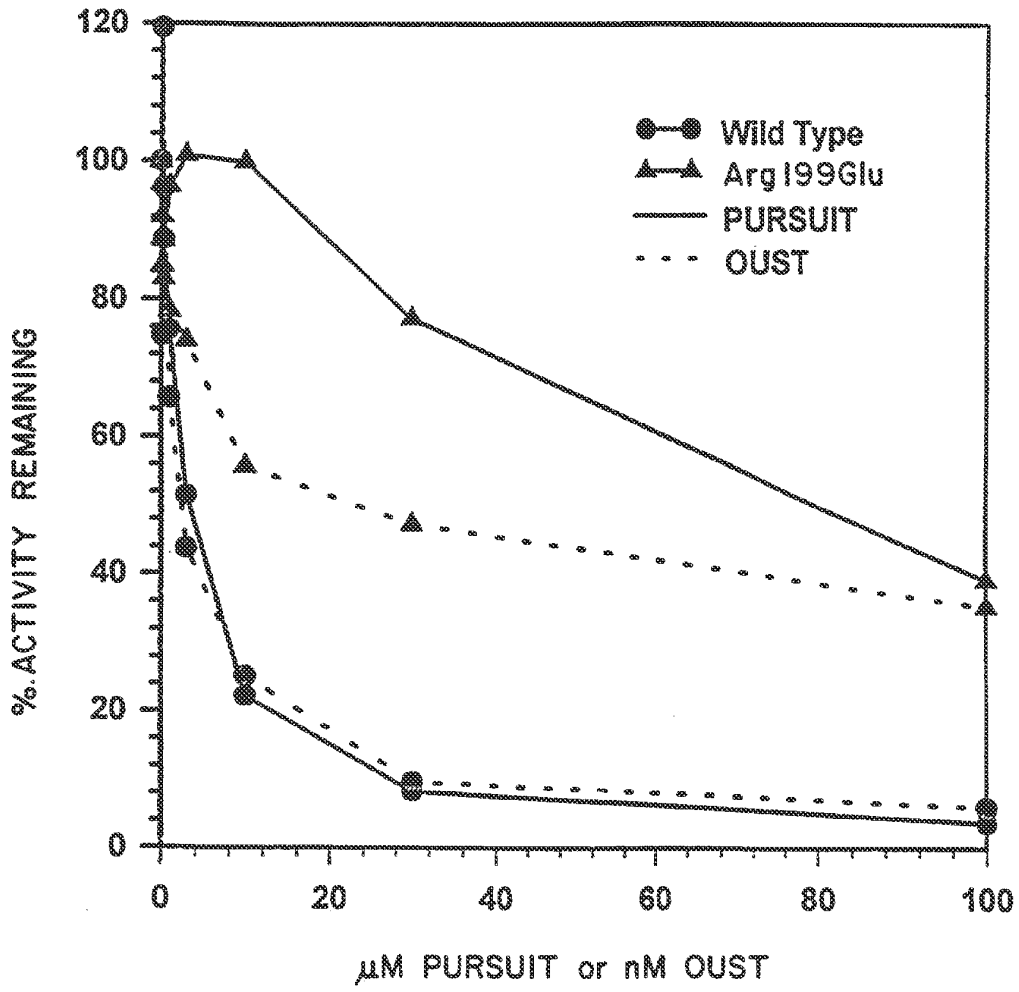
COR-AAD1-00004272
Bayer EX1008 (Part 8 of 9)

FIG. 10



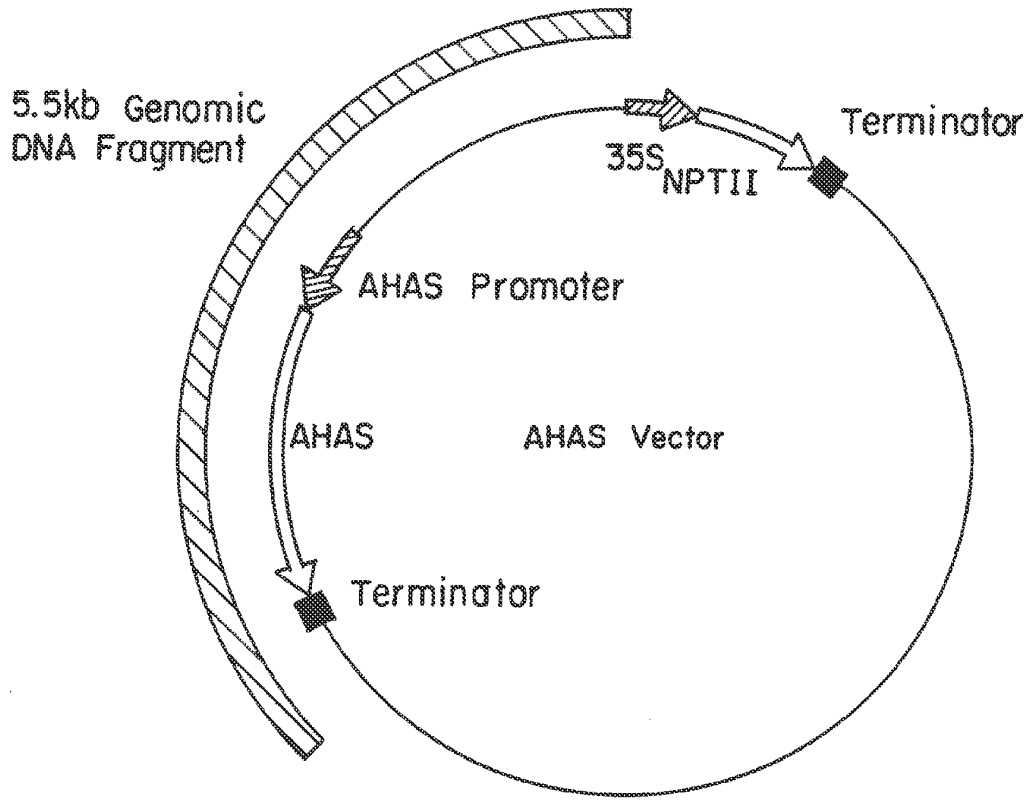
COR-AAD1-00004273
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FIG. 11



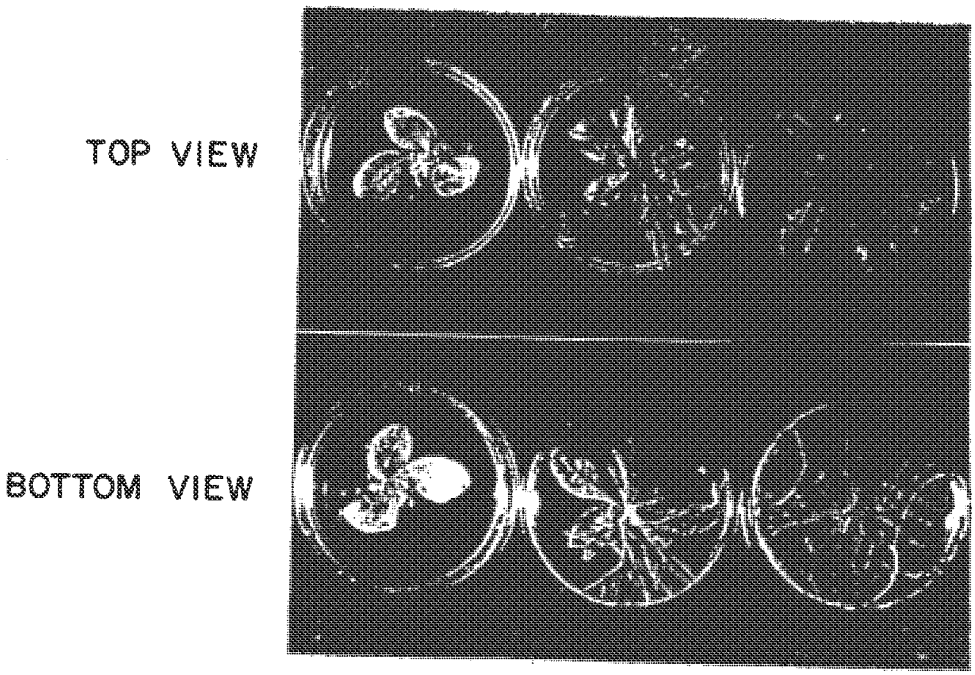
COR-AAD1-00004274
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FIG. 12



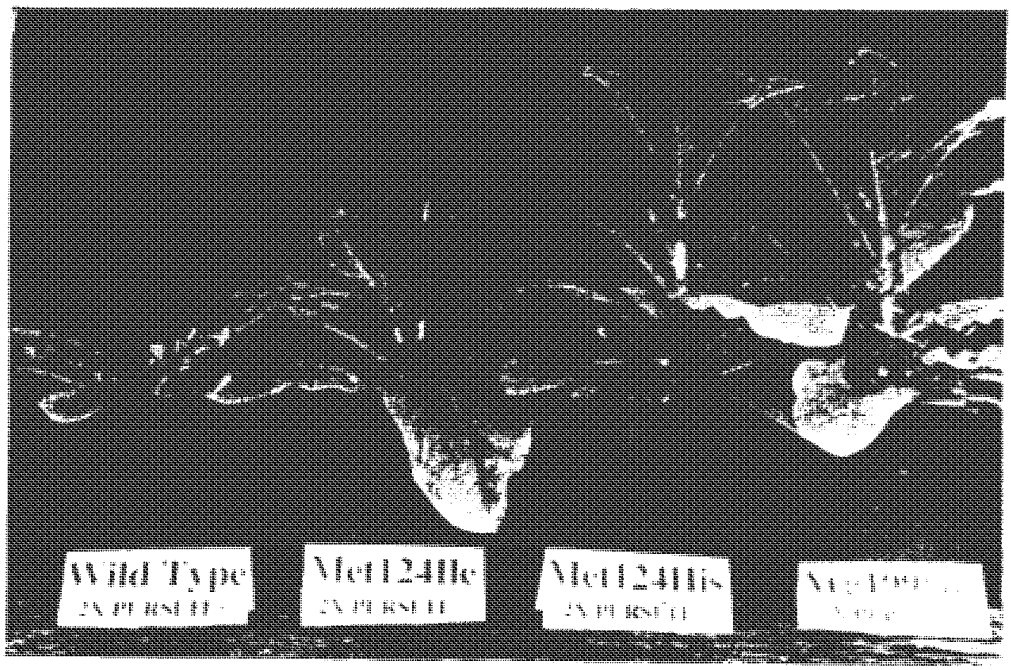
COR-AAD1-00004275
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FIG. 13



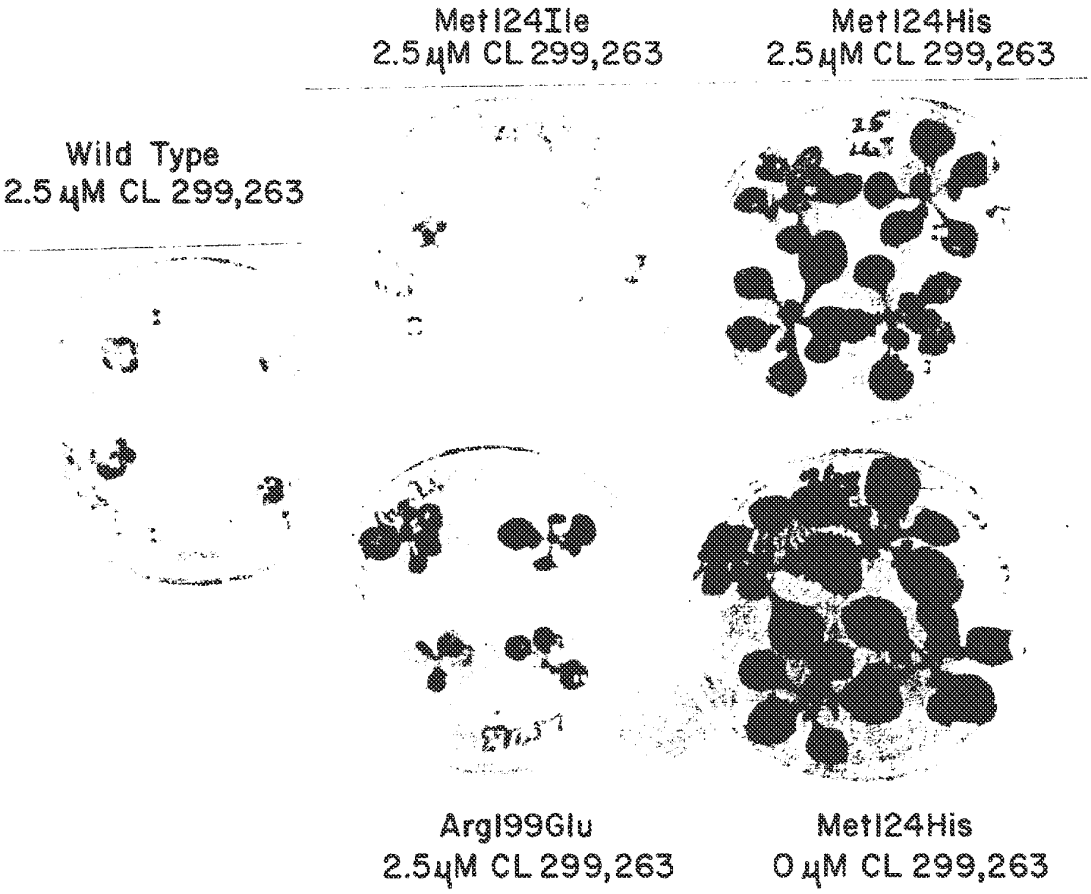
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FIG. 14



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FIG. 15



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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05782

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 15/00, 9/88; A01H 5/00, 5/10
US CL :435/172.3, 232; 800/205
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/172.3, 232; 800/205

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, STN: medline, caplus, scisearch, lifesci, biosis, embase, wpids, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	MOSIMANN et al. A Critical Assessment of Comparative Molecular Modeling of Tertiary Structure of Proteins. Proteins: Structure, Function and Genetics November 1995, Vol. 23, pages 301-317. See from the beginning of the discussion section on page 311 to the end on page 316.	1-32
X --- A	BLUNDELL et al. Knowledge-based prediction of protein structures and the design of novel molecules. Nature 26 March 1987, Vol. 326, pages 347-352. See the entire article and in particular page 348, left column.	1-32 --- 70-82

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

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

Date of the actual completion of the international search 21 AUGUST 1996	Date of mailing of the international search report 18 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NASHED Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05782

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Protein Science, Vol. 3, Suppl. 1, July 1994, page 135, right column, the abstract number 479-S, IBDAH et al. MODEL FOR THE ACTIVE SITE OF ACETYLHYDROXY ACID SYNTHASE BASED ON HOMOLOGY MODELING AND DIRECTED MUTAGENESIS.	1-32 -------- 70-82
X, P ------ Y, P	BERNASCONI et al. A Naturaly Occurring Mutation Confers Broad Range Tolerance to Heribicde That Target Acetolactate Synthase. Journal of Biological Chemistry, 21 July 1995, Vol. 270, No. 29, pages 17381-178385. See abstract.	61, 62, 64 ---------- 1-57, 65-69
X --- Y	YADAV et al. Single amino acid substitutions in the enzyme acetolactate synthase confer resistance to the herbicide sulfometuron methyl. Proc. Natl. Acad. Sci. USA, June 1986, Vol. 83, pages 4418-4422. See particularly Figure 2 on page 4420.	61, 62, 64 ---------- 1-57, 65-69
X --- Y	SATHASIVA et al. Nucleotide sequence of a mutant acetolactate synthase gene from an imidazolinone-resitant Arabidopsis thaliana var. Columbia. Nucleic Acids Research 1990, Vol. 18, No. 8, page 2188. The entire article.	63 ---------- 18, 28, 36, 49, 57
X --- Y	US 5,013,659 A (BEDBROOK et al.) 07 May 1991. The entire document.	61, 62 ---------- 1-60, 63, 83, 84
Y	HAUGHN et al. Sulfonylurea-resistant mutants of Arabidopsis thaliana. Mol Gen Genet,1986, Vol. 204, pages 430-434. See abstract.	18, 28, 36, 49, 57, 63
X --- Y	HATTORI et al. Multiple resistance to sulfonylureas and imidazolinones conferred by an acetylhydroxyacid synthase gene with separate mutations for selective resistance. Mol Gen Genet. Vol. 232, 1992, pages 167-173. See entire article.	61-64 ---------- 1-60, 66-69, 83, 84
A	STIDHAM, Herbicides that Inhibit Acetohydroxyacid Synthase. Weed Science 1991, Vol. 39, 428-434.	70-82
Y	LAM et al. Rational Design of Potent, Bioavailable, Nonpeptide Cyclic Ureas as HIV Protease Inhibitors Science, 21 January 1994, Vol. 263, pages 380-384.	70-82
Y	WHITTLE et al. PROTEIN STRUCTURE-BASED DRUG DESIGN. Annu. Rev. Biophys. Biomol. Struct. 1994, Vol. 23, pages 349-375. The entire article.	70-82

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05782

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MULLER et al. Structure of Tiamine- and Flavin-Dependent Enzyme Pyruvate Oxidase. Science, 12 February 1993, Vol. 259, pages 965-967. The entire article.	1-32, 70-82

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05782

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05782

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-43, 45-54, 56-67 and 83, drawn to a methods for the design of herbicide-resistant acetohydroxy acid synthase (AHAS), the mutants herbicide-resistant AHAS, their DNA sequences, vectors containing the DNA sequences and the cells containing the expression vectors.

Group II, claims 44, 55, 68, 69, and 84, drawn to herbicide-resistant plants and seeds and a method of controlling the growth of weeds.

Group III, claims 70-82, drawn to a method to design herbicides.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I comprises a method of protein/enzyme engineering to produce mutant enzymes that have full enzymatic activity, but have a reduced or no affinity to bind inhibitors of the wild-type enzyme. The special technical feature in the method of Group I is the protein design step, whereas that of Group II is the design of a small molecule that can bind best to the wild-type enzyme. Both special technical features of Groups I and II are different from that of Group III which is a herbicide resistant plants and seeds. Thus, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.1 so as to form a single invention concept.

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(54) Corn transformant PV-ZMGT32 (NK603) and compositions and methods for detection thereof

(57) The present invention provides a DNA construct that confers tolerance to transgenic corn plant. Also provided are assays for detecting the presence of the PV-ZMGT32(nk603) corn event based on the DNA sequence of the recombinant construct inserted into the corn genome and of genomic sequences flanking the insertion site.

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Description

FIELD OF THE INVENTION

[0001] The present invention relates to the field of plant molecular biology, specifically the invention relates to a DNA construct for conferring glyphosate tolerance to a plant. The invention more specifically relates to a glyphosate tolerant corn plant PV-ZMGT32(nk603) and to assays for detecting the presence of corn plant PV-ZMGT32(nk603) DNA in a sample and compositions thereof.

BACKGROUND OF THE INVENTION

[0002] This invention relates to the glyphosate herbicide tolerant corn (*Zea mays*) plant PV-ZMGT32(nk603) and to the DNA plant expression construct of corn plant PV-ZMGT32(nk603) and the detection of the transgene/genomic insertion region in corn PV-ZMGT32(nk603) and progeny thereof.

[0003] Corn is an important crop and is a primary food source in many areas of the world. The methods of biotechnology have been applied to corn for improvement of the agronomic traits and the quality of the product. One such agronomic trait is herbicide tolerance, in particular, tolerance to glyphosate herbicide. This trait in corn has been conferred by the expression of a transgene in the corn plants (U.S. Patent No. 6,040,497).

[0004] The expression of foreign genes in plants is known to be influenced by their chromosomal position, perhaps due to chromatin structure (*e.g.*, heterochromatin) or the proximity of transcriptional regulation elements (*e.g.*, enhancers) close to the integration site (Weising *et al.*, Ann. Rev. Genet 22:421-477, 1988). For this reason, it is often necessary to screen a large number of events in order to identify an event characterized by optimal expression of a introduced gene of interest. For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of introduced genes among events. There may also be differences in spatial or temporal patterns of expression, for example, differences in the relative expression of a transgene in various plant tissues, that may not correspond to the patterns expected from transcriptional regulatory elements present in the introduced gene construct. For this reason, it is common to produce hundreds to thousands of different events and screen those events for a single event that has desired transgene expression levels and patterns for commercial purposes. An event that has desired levels or patterns of transgene expression is useful for introgressing the transgene into other genetic backgrounds by sexual outcrossing using conventional breeding methods. Progeny of such crosses maintain the transgene expression characteristics of the original transformant. This strategy is used to ensure reliable gene expression in a number of varieties that are well adapted to local growing conditions.

[0005] It would be advantageous to be able to detect the presence of a particular event in order to determine whether progeny of a sexual cross contain a transgene of interest. In addition, a method for detecting a particular event would be helpful for complying with regulations requiring the premarket approval and labeling of foods derived from recombinant crop plants, for example. It is possible to detect the presence of a transgene by any well known nucleic acid detection method such as the polymerase chain reaction (PCR) or DNA hybridization using nucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, terminators, marker genes, etc. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct unless the DNA sequence of chromosomal DNA adjacent to the inserted DNA ("flanking DNA") is known. An event-specific PCR assay is discussed, for example, by Windels *et al.* (Med. Fac. Landbouww, Univ. Gent 64/5b:459-462, 1999), who identified glyphosate tolerant soybean event 40-3-2 by PCR using a primer set spanning the junction between the insert and flanking DNA, specifically one primer that included sequence from the insert and a second primer that included sequence from flanking DNA.

SUMMARY OF THE INVENTION

[0006] According to one aspect of the invention, a DNA construct is provided that when expressed in plant cells and plants confers tolerance to glyphosate herbicide. This invention relates preferably to the methods for producing and selecting a glyphosate tolerant monocot crop plant. The DNA construct consists of two transgene expression cassettes. The first expression cassette comprising a DNA molecule of a rice (*Oryzae sativa*) actin 1 promoter and rice actin 1 intron operably joined to a DNA molecule encoding a chloroplast transit peptide sequence, operably connected to a DNA molecule encoding a glyphosate resistant 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS), operably connected to a DNA molecule comprising a 3' transcriptional terminator. The second transgene expression cassette of the DNA construct comprising a DNA molecule of the cauliflower mosaic virus (CaMV) 35S promoter, operably connected to a DNA molecule comprising a Hsp70 intron, operably connected to a DNA molecule encoding a chloroplast transit peptide sequence, operably connected to a DNA molecule encoding a glyphosate resistant 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS), operably connected to a DNA molecule comprising a 3' transcriptional termi-

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nator.

[0007] More specifically, a DNA construct is provided that when expressed in plant cells and plants confers tolerance to glyphosate herbicide. This invention relates preferably to the methods for producing and selecting a glyphosate tolerant corn plant. The DNA construct consists of two transgene expression cassettes. The first expression cassette consisting of a DNA molecule of a rice (*Oryzae sativa*) actin 1 promoter and rice actin 1 intron operably joined to a DNA molecule encoding an *Arabidopsis* EPSPS chloroplast transit peptide sequence, operably connected to a DNA molecule encoding a glyphosate resistant 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) isolated from *Agrobacterium tumefaciens* sp. strain CP4, operably connected to a DNA molecule consisting of a nopaline synthase transcriptional terminator. The second transgene expression cassette consisting of a DNA molecule of the cauliflower mosaic virus (CaMV) 35S promoter containing a tandem duplication of the enhancer region, operably connected to a DNA molecule consisting of a *Zea mays* Hsp70 intron, operably connected to a DNA molecule encoding an *Arabidopsis* EPSPS chloroplast transit peptide sequence, operably connected to a DNA molecule encoding a glyphosate resistant 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) isolated from *Agrobacterium tumefaciens* sp. strain CP4, operably connected to a DNA molecule consisting of a nopaline synthase transcriptional terminator.

[0008] According to another aspect of the invention, compositions and methods are provided for detecting the presence of the transgene/genomic insertion region from a novel corn plant designated PV-ZMGT32(nk603). DNA molecules are provided that comprise at least one junction sequence of PV-ZMGT32(nk603) selected from the group consisting of

5' TGTAGCGGCCCACGCGTGG 3' (SEQ ID NO:9), 5' TACCACGCGACACACTTC 3' (SEQ ID NO:10), and 5' TGCTGTTCTGCTGACTTT 3' (SEQ ID NO:11) and complements thereof; wherein a junction sequence spans the junction between heterologous DNA inserted into the genome and the DNA from the corn cell flanking the insertion site and is diagnostic for the event. The corn plant and seed comprising these molecules is an aspect of this invention.

[0009] A novel DNA molecule 5'ACCAAGCTTTTATAATAG 3' (SEQ ID NO:12) and the complement thereof, wherein this DNA molecule is novel in PV-ZMGT32(nk603) and its progeny, the corn plant and seed comprising this molecule are other aspects of this invention.

[0010] According to another aspect of the invention, DNA molecules that comprise the novel transgene/genomic insertion region, SEQ ID NO:7 and SEQ ID NO:8 and are homologous or complementary to SEQ ID NO:7 and SEQ ID NO:8 are an aspect of this invention.

[0011] DNA molecules that comprise a sufficient length of a transgene portion of the DNA sequence of SEQ ID NO: 7 and a similar sufficient length of a 5' flanking corn DNA sequence of SEQ ID NO:7; or a similar sufficient length of a transgene portion of the DNA sequence of SEQ ID NO:8 and a similar sufficient length of a 3' DNA sequence flanking the transgene, wherein these DNA molecules are useful as DNA primers in DNA amplification methods so as to provide a DNA amplicon product specifically produced from PV-ZMGT32(nk603) DNA and its progeny are another aspect of the invention. DNA primers homologous or complementary to a length of SEQ ID NO:7 and SEQ ID NO:8 are an aspect of the invention. The amplicons produced using DNA primers that are diagnostic for corn event PV-ZMGT32(nk603) and its progeny are a subject of this invention.

[0012] According to another aspect of the invention, methods of detecting the presence of DNA corresponding to the corn event PV-ZMGT32(nk603) event in a sample are provided. Such methods comprise: (a) contacting the sample comprising DNA with a DNA primer set, that when used in a nucleic acid amplification reaction with genomic DNA extracted from corn event PV-ZMGT32 (nk603) produces an amplicon that is diagnostic for corn event PV-ZMGT32 (nk603); (b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and (c) detecting the amplicon. A pair of DNA molecules comprising a DNA primer set that are homologous or complementary to SEQ ID NO:7 or SEQ ID NO:8 that function in a nucleic acid amplification reaction to produce an amplicon DNA molecule diagnostic for PV-ZMGT329(nk603) are provided. More specifically, a pair of DNA molecules comprising a DNA primer set, wherein the DNA molecules are identified as SEQ ID NO:13 or complements thereof and SEQ ID NO:14 or complements thereof; SEQ ID NO:15 or complements thereof and SEQ ID NO:16 or complements thereof are provided. The amplicon comprising the DNA molecules of SEQ ID NO:13 and SEQ ID NO:14. The amplicon comprising the DNA molecules of SEQ ID NO:15 and SEQ ID NO:16. The amplicon produced by the afore described method can hybridize under stringent conditions to SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, or SEQ ID NO:12.

[0013] According to another aspect of the invention, methods of detecting the presence of a DNA molecule corresponding to the PV-ZMGT32(nk603) event in a sample, such methods comprising: (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe molecule that hybridizes under stringent hybridization conditions with genomic DNA from corn event PV-ZMGT32(nk603) and does not hybridize under the stringent hybridization conditions with a control corn plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA. More specifically, a method for detecting the presence of a DNA molecule corresponding to the PV-ZMGT32(nk603) event in a sample, such methods, consisting of (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe molecule that consists of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, or SEQ ID NO:12, wherein said DNA probe molecule hybridizes under stringent hybridization

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conditions with genomic DNA from corn event PV-ZMGT32(nk603) and does not hybridize under the stringent hybridization conditions with a control corn plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA.

[0014] According to another aspect of the invention, methods of producing a corn plant that tolerates application of glyphosate are provided that comprise the steps of: (a) sexually crossing a first parental corn line comprising the expression cassettes of the present invention, which confers tolerance to application of glyphosate, and a second parental corn line that lacks the glyphosate tolerance, thereby producing a plurality of progeny plants; and (b) selecting a progeny plant that tolerates application of glyphosate. Such methods may optionally comprise the further step of back-crossing the progeny plant to the second parental corn line to producing a true-breeding corn plant that tolerates application of glyphosate.

[0015] According to another aspect of the invention, a method is provided to select for glyphosate tolerant corn plants of the present invention and progeny thereof comprising extracting DNA from a plant sample, contacting a DNA with a marker nucleic acid molecule selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, or SEQ ID NO:12, or complements thereof, detecting the hybridization of said marker nucleic acid molecule to the DNA, and performing a marker assisted breeding analysis for the genetic linkage of the glyphosate tolerant trait to the marker nucleic acid molecule.

[0016] The present invention provides a method of producing a corn plant tolerant to glyphosate herbicide comprising transforming a corn cell with the DNA construct (pMON25496), selecting the corn cell for tolerance to the treatment with an effective dose of glyphosate, and growing the corn cell into a fertile corn plant. The fertile corn plant can be self pollinated or crossed with compatible corn varieties to produce glyphosate tolerant progeny.

[0017] The invention further relates to a DNA detection kit comprising at least one DNA molecule of sufficient length of contiguous nucleotides homologous or complementary to SEQ ID NO:7 or SEQ ID NO:8 that functions as a DNA primer or probe specific for corn event PV-ZMGT32(nk603) and/or its progeny.

[0018] This invention further relates to the plants and seeds of glyphosate tolerant corn (*Zea mays*) PV-ZMGT32 (nk603) having ATCC Accession No. PTA-2478 and the progeny derived thereof. The corn plant or its parts produced by growing of the glyphosate tolerant corn plant PV-ZMGT32(nk603), the pollen and ovules of the corn plant PV-ZMGT32(nk603). The nuclei of vegetative cells, the nuclei of pollen cells, and the nuclei of egg cells of the corn plant PV-ZMGT32 (nk603) and the progeny derived thereof. The corn plant and seed PV-ZMGT32(nk603) from which the DNA primer molecules of the present invention provide a specific amplicon product is an aspect of the invention.

[0019] The foregoing and other aspects of the invention will become more apparent from the following detailed description and accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figure 1. Plasmid map of pMON25496

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0021] The following definitions and methods are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. Definitions of common terms in molecular biology may also be found in Rieger *et al.*, *Glossary of Genetics: Classical and Molecular,* 5th edition, Springer-Verlag: New York, 1991; and Lewin, *Genes V*, Oxford University Press: New York, 1994. The nomenclature for DNA bases as set forth at 37 CFR § 1.822 is used.

[0022] As used herein, the term "corn" means *Zea mays* or maize and includes all plant varieties that can be bred with corn, including wild maize species.

[0023] As used herein, the term "comprising" means "including but not limited to".

[0024] "Glyphosate" refers to N-phosphonomethylglycine and its salts, Glyphosate is the active ingredient of Roundup® herbicide (Monsanto Co.). Treatments with "glyphosate herbicide" refer to treatments with the Roundup®, Roundup Ultra®, Roundup UltraMax® herbicide or any other herbicide formulation containing glyphosate. The selection of application rates for a glyphosate formulation that constitute a biologically effective dose is within the skill of the ordinary agricultural technician.

[0025] A DNA construct is an assembly of DNA molecules linked together that provide one or more expression cassettes. The DNA construct is preferably a plasmid that is enabled for self replication in a bacterial cell and contains various endonuclease enzyme restriction sites that are useful for introducing DNA molecules that provide functional genetic elements, *i.e.*, promoters, introns, leaders, coding sequences, 3' termination regions, among others. The expression cassettes contained within a DNA construct comprise the necessary genetic elements to provide transcription of a messenger RNA. The expression cassettes can be designed to express in prokaryote cells or eukaryotic cells.

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The expression cassettes of the present invention are designed to express most preferably in plant cells. The combination of expression cassettes in transgenic plants can lead to unexpected phenotypes. In the present invention, the combination of the rice actin 1 promoter cassette and the CaMV 35S promoter cassette provided in a DNA construct (pMON25496) enhanced transgenic corn plant tolerance to glyphosate herbicide when compared to the single cassettes alone and to the commercial standard so-called GA21 that contains three copies of a rice actin 1 promoter cassette.

[0026] A transgenic "event" is produced by transformation of plant cells with heterologous DNA construct, including a nucleic acid expression cassette that comprises a transgene of interest, the regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant, and selection of a particular plant characterized by insertion into a particular genome location. The term "event" refers to the original transformant and progeny of the transformant that include the heterologous DNA. The term "event" also refers to progeny produced by a sexual outcross between the transformant and another variety that include the heterologous DNA. Even after repeated back-crossing to a recurrent parent, the inserted DNA and flanking DNA from the transformed parent is present in the progeny of the cross at the same chromosomal location. The term "event" also refers to DNA from the original transformant comprising the inserted DNA and flanking genomic sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA. A glyphosate tolerant corn plant PV-ZMGT32 (nk603) can be breed by first sexually crossing a first parental corn plant consisting of a corn plant grown from the transgenic corn plant PV-ZMGT32 (nk603) having ATCC Accession No. PTA-2478 and progeny thereof derived from transformation with the expression cassettes of the present invention that tolerates application of glyphosate herbicide, and a second parental corn plant that lacks the tolerance to glyphosate herbicide, thereby producing a plurality of first progeny plants; and then selecting a first progeny plant that is tolerant to application of glyphosate herbicide; and selfing the first progeny plant, thereby producing a plurality of second progeny plants; and then selecting from the second progeny plants a glyphosate herbicide tolerant plant. These steps can further include the back-crossing of the first glyphosate tolerant progeny plant or the second glyphosate tolerant progeny plant to the second parental corn plant or a third parental corn plant, thereby producing a corn plant that tolerates the application of glyphosate herbicide.

[0027] It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in one of several references, e.g., Fehr, in *Breeding Methods for Cultivar Development,* Wilcox J. ed., American Society of Agronomy, Madison WI (1987).

[0028] A "probe" is an isolated nucleic acid to which is attached a conventional detectable label or reporter molecule, e.g., a radioactive isotope, ligand, chemiluminescent agent, or enzyme. Such a probe is complementary to a strand of a target nucleic acid, in the case of the present invention, to a strand of genomic DNA from corn event PV-ZMGT32 (nk603) whether from a corn plant or from a sample that includes DNA from the event. Probes according to the present invention include not only deoxyribonucleic or ribonucleic acids but also polyamides and other probe materials that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

[0029] "Primers" are isolated nucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, *e.g.,* a DNA polymerase. Primer pairs of the present invention refer to their use for amplification of a target nucleic acid sequence, *e.g.,* by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods.

[0030] Probes and primers are of sufficient nucleotide length to bind to the target DNA sequence specifically in the hybridization conditions or reaction conditions determined by the operator. This length may be of any length that is of sufficient length to be useful in the detection method of choice. Generally, 11 nucleotides or more in length, preferably 18 nucleotides or more, more preferably 24 nucleotides or more, and most preferably 30 nucleotides or more are used. Such probes and primers hybridize specifically to a target sequence under high stringency hybridization conditions. Preferably, probes and primers according to the present invention have complete DNA sequence similarity of contiguous nucleotides with the target sequence, although probes differing from the target DNA sequence and that retain the ability to hybridize to target DNA sequences may be designed by conventional methods.

[0031] Methods for preparing and using probes and primers are described, for example, in *Molecular Cloning: A Laboratory Manual,* 2nd ed., vol. 1-3, ed. Sambrook *et al.,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989 (hereinafter, "Sambrook *et al.,* 1989"); *Current Protocols in Molecular Biology,* ed. Ausubel *et al.*, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates) (hereinafter, "Ausubel *et al.*, 1992"); and Innis *et al.*, *PCR Protocols: A Guide to Methods and Applications*, Academic Press: San Diego, 1990. PCR-primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose

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such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

[0032] Primers and probes based on the flanking DNA and insert sequences disclosed herein can be used to confirm (and, if necessary, to correct) the disclosed sequences by conventional methods, *e.g.,* by re-cloning and sequencing such sequences.

[0033] The nucleic acid probes and primers of the present invention hybridize under stringent conditions to a target DNA sequence. Any conventional nucleic acid hybridization or amplification method can be used to identify the presence of DNA from a transgenic event in a sample. Nucleic acid molecules or fragments thereof are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure. A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook *et al.*, 1989, and by Haymes *et al.*, In: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985), Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

[0034] As used herein, a substantially homologous sequence is a nucleic acid molecule that will specifically hybridize to the complement of the nucleic acid molecule to which it is being compared under high stringency conditions. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology,* John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed. In a preferred embodiment, a nucleic acid of the present invention will specifically hybridize to one or more of the nucleic acid molecules set forth in SEQ ID NO: 9, 10, 11 and 12 or complements thereof or fragments of either under moderately stringent conditions, for example at about 2.0 x SSC and about 65°C. In a particularly preferred embodiment, a nucleic acid of the present invention will specifically hybridize to one or more of the nucleic acid molecules set forth in SEQ ID NO:9 through SEQ ID NO:12 or complements or fragments of either under high stringency conditions. In one aspect of the present invention, a preferred marker nucleic acid molecule of the present invention has the nucleic acid sequence set forth in SEQ ID NO:9 through SEQ ID NO:12 or complements thereof or fragments of either. In another aspect of the present invention, a preferred marker nucleic acid molecule of the present invention shares between 80% and 100% or 90% and 100% sequence identity with the nucleic acid sequence set forth in SEQ ID NO:9 through SEQ ID NO:12 or complement thereof or fragments of either. In a further aspect of the present invention, a preferred marker nucleic acid molecule of the present invention shares between 95% and 100% sequence identity with the sequence set forth in SEQ ID NO:9 through SEQ ID NO:12 or complement thereof or fragments of either. SEQ ID NO:9 through SEQ IN NO:12 may be used as markers in plant breeding methods to identify the progeny of genetic crosses similar to the methods described for simple sequence repeat DNA marker analysis, in "DNA markers: Protocols, applications, and overviews: (1997) 173-185, Cregan, et al., eds., Wiley-Liss NY; all of which is herein incorporated by reference in its' entirely. The hybridization of the probe to the target DNA molecule can be detected by any number of methods known to those skilled in the art, these can include, but are not limited to, fluorescent tags, radioactive tags, antibody based tags, and chemiluminescent tags.

[0035] Regarding the amplification of a target nucleic acid sequence (*e.g.*, by PCR) using a particular amplification primer pair, "stringent conditions" are conditions that permit the primer pair to hybridize only to the target nucleic-acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce a unique amplification product, the amplicon, in a DNA thermal amplification reaction.

[0036] The term "specific for (a target sequence)" indicates that a probe or primer hybridizes under stringent hybridization conditions only to the target sequence in a sample comprising the target sequence.

[0037] As used herein, "amplified DNA" or "amplicon" refers to the product of nucleic-acid amplification of a target nucleic acid sequence that is part of a nucleic acid template. For example, to determine whether the corn plant resulting from a sexual cross contains transgenic event genomic DNA from the corn plant of the present invention, DNA extracted from a corn plant tissue sample may be subjected to nucleic acid amplification method using a DNA primer pair that

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includes a first primer derived from flanking sequence in the genome of the plant adjacent to the insertion site of inserted heterologous DNA, and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the event DNA. The amplicon is of a length and has a sequence that is also diagnostic for the event. The amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair, preferably plus about fifty nucleotide base pairs, more preferably plus about two hundred-fifty nucleotide base pairs, and even more preferably plus about four hundred-fifty nucleotide base pairs. Alternatively, a primer pair can be derived from flanking sequence on both sides of the inserted DNA so as to produce an amplicon that includes the entire insert nucleotide sequence (*e.g.*, the MluI DNA fragment of the pMON25496 expression construct, Figure 1, approximately 6706 nucleotide base pairs). A member of a primer pair derived from the plant genomic sequence may be located a distance from the inserted DNA sequence, this distance can range from one nucleotide base pair up to the limits of the amplification reaction, or about twenty thousand nucleotide base pairs. The use of the term "amplicon" specifically excludes primer dimers that may be formed in the DNA thermal amplification reaction.

[0038] Nucleic-acid amplification can be accomplished by any of the various nucleic-acid amplification methods known in the art, including the polymerase chain reaction (PCR). A variety of amplification methods are known in the art and are described, *inter alia,* in U.S. Patent Nos. 4,683,195 and 4,683,202 and in *PCR Protocols: A Guide to Methods and Applications,* ed. Innis *et al.,* Academic Press, San Diego, 1990. PCR amplification methods have been developed to amplify up to 22 kb of genomic DNA and up to 42 kb of bacteriophage DNA (Cheng *et al.,* Proc. Natl. Acad. Sci. USA 91:5695-5699, 1994). These methods as well as other methods known in the art of DNA amplification may be used in the practice of the present invention. The sequence of the heterologous DNA insert or flanking DNA sequence from corn event PV-ZMGT32(nk603) can be verified (and corrected if necessary) by amplifying such sequences from DNA extracted from the ATCC deposit Accession No. PTA-2478 seed or plants using DNA primers derived from the sequences provided herein followed by standard DNA sequencing of the PCR amplicon or of the cloned DNA.

[0039] The amplicon produced by these methods may be detected by a plurality of techniques. One such method is Genetic Bit Analysis (Nikiforov, *et al.* Nucleic Acid Res. 22:4167-4175, 1994) where an DNA oligonucleotide is designed which overlaps both the adjacent flanking genomic DNA sequence and the inserted DNA sequence . The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking genomic sequence), a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labelled ddNTPs specific for the expected next base. Readout may be fluorescent or ELISA-based. A signal indicates presence of the insert/flanking sequence due to successful amplification, hybridization, and single base extension.

[0040] Another method is the pyrosequencing technique as described by Winge (Innov. Pharma. Tech. 00:18-24, 2000). In this method an oligonucleotide is designed that overlaps the adjacent genomic DNA and insert DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking genomic sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. dNTPs are added individually and the incorporation results in a light signal which is measured. A light signal indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single or multi-base extension.

[0041] Fluorescence Polarization as described by Chen, *et al.*, (Genome Res. 9:492-498, 1999) is a method that can be used to detect the amplicon of the present invention. Using this method an oligonucleotide is designed which overlaps the genomic flanking and inserted DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted DNA and one in the flanking genomic DNA sequence) and incubated in the presence of a DNA polymerase and a fluorescent-labeled ddNTP. Single base extension results in incorporation of the ddNTP. Incorporation can be measured as a change in polarization using a fluorometer. A change in polarization indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single base extension.

[0042] Taqman® (PE Applied Biosystems, Foster City, CA) is described as a method of detecting and quantifying the presence of a DNA sequence and is fully understood in the instructions provided by the manufacturer. Briefly, a FRET oligonucleotide probe is designed which overlaps the genomic flanking and insert DNA junction. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Hybridization of the FRET probe results in cleavage and release of the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

[0043] Molecular Beacons have been described for use in sequence detection as described in Tyangi, *et al.* (Nature Biotech.14:303-308, 1996) Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking genomic and insert DNA junction. The unique structure of the FRET probe results in it containing secondary structure that keeps the fluorescent and quenching moieties in close proximity. The FRET probe and PCR primers (one primer in the insert

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DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Following successful PCR amplification, hybridization of the FRET probe to the target sequence results in the removal of the probe secondary structure and spatial separation of the fluorescent and quenching moieties. A fluorescent signal results. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

[0044] The following examples are included to demonstrate examples of certain preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to constitute examples of preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

EXAMPLE 1

[0045] The PV-ZMGT32(nk603) (hence forth referred to as nk603) transgenic corn event was generated by microprojectile bombardment of maize embryos (Songstad *et al.*, In Vitro Cell Plant 32:179-183, 1996) using a linear Mlu I DNA fragment derived from pMON25496 (Figure 1). This DNA fragment contains two transgene expression cassettes that collectively confer corn plant tolerance to glyphosate. The first cassette is composed of the rice actin 1 promoter and intron (P-Os.Act1 and I-Os.Act1, U.S. Patent No. 5,641,876), operably connected to an *Arabidopsis* EPSPS chloroplast transit peptide (TS-At.EPSPS:CTP2, Klee *et al.*, Mol. Gen. Genet. 210:47-442, 1987), operably connected to a glyphosate tolerant 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) from *Agrobacterium sp.* strain CP4 (AGRTU.*aroA*:CP4, U.S. Patent No. 5,633,435) and operably connected to a nopaline synthase transcriptional terminator (T-AGRTU.nos, Fraley *et al.* Proc. Natl. Acad. Sci. USA 80:4803-4807, 1983). The second transgene expression cassette consists of the cauliflower mosaic virus 35S promoter containing a tandem duplication of the enhancer region (P-CaMV.35S, Kay *et al.* Science 236:1299-1302, 1987; U.S. Patent No. 5,164,316), operably connected to a *Zea mays* Hsp70 intron (I-Zm.Hsp70, U.S. Patent No. 5,362,865), operably connected to an *Arabidopsis* EPSPS chloroplast transit peptide (TS-At.EPSPS:CTP2, Klee *et al.*, Mol. Gen. Genet. 210:47-442, 1987), operably connected to a glyphosate tolerant 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) from *Agrobacterium sp.* strain CP4 (AGRTU.*aroA*:CP4, U.S. Patent No. 5,633,435) and operably connected to a nopaline synthase transcriptional terminator (T-AGRTU.nos, Fraley *et al.* Proc. Natl. Acad. Sci. USA 80:4803-4807, 1983). Post-bombardment, glyphosate-tolerant transgenic calli were selected on media containing 3 mM glyphosate and plants were subsequently regenerated. Three hundred-four plants from 91 independent transgenic events were produced, nk603 was selected from this population based on a superior combination of characteristics, including glyphosate tolerance, agronomic performance, and single transgenic insertion. Greenhouse and field evaluations of the nk603 event and its derived progeny indicated this transgenic insertion confers tolerance that exceeds commercial specifications for full vegetative and reproductive tolerance to 340 g glyphosate/acre (840 g glyphosate/hectare; 32 oz Roundup Ultra/acre) when applied at the V4 and V8 leaf stages.

EXAMPLE 2

[0046] The glyphosate tolerant nk603 corn event was compared to the current commercial standard, GA21 (U.S. Patent No. 6,040,497), for tolerance to glyphosate vegetative injury and effect on yield. GA21 contains at least 3 transgene expression cassettes arranged in tandem in the corn genome of GA21 event (SCP/GMO/232-Final, European Commission Health & Consumer Protection Directorate-General). The transgene cassette of GA21 consists of a rice actin 1 promoter and intron linked to a ribulose 1,5-bisphosphate carboxylase chloroplast transit peptide linked to a modified maize glyphosate resistant EPSPS and a 3' nopaline synthase transcription termination region. Plants of nk603 and GA21 were planted in rows in replicated field plots. The treatments were 1) unsprayed, 2) sprayed at a 64 ounces/acre rate of Roundup Ultra® at V4 leaf stage and again with 64 ounces/acre Roundup Ultra® at V8 leaf stage, 3) sprayed at a 96 ounces/acre rate of Roundup Ultra® at V4 leaf stage and again with 96 ounces/acre Roundup Ultra® at V8 leaf stage. Vegetative tolerance was measured as percent vegetative injury determined by the amount of leaf malformation observed 10 days after the herbicide treatment at the V8 leaf stage. The yield of each plot was measured in bushels/acre and the percent reduction in yield determined for each herbicide treatment relative to the unsprayed treatment. The results shown in Table 1 illustrates that nk603 shows a lower percent vegetative injury level than the GA21 plants and the observed percent yield reduction is also less for the nk603 event. A low incidence of vegetative injury was observed in the unsprayed plots, this observation is attributable to various environmental factors other than glyphosate herbicide exposure. The double expression cassette of pMON25496 in nk603 was compared to vegetative

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injury and fertility rating of 3 independent corn events obtained from only the CaMV.35S promoter driving expression of the glyphosate tolerance gene (AGRTU.aroA:CP4). It was observed that the double expression cassette conferred a higher level of vegetative tolerance and reproductive tolerance than three independent corn events (ev 1, ev 2, and ev 3) containing only the expression cassette where the glyphosate tolerance gene expression was driven by the CaMV.35S promoter. A higher level of vegetative tolerance to glyphosate herbicide injury was observed for nk603 plus 3 additional corn events derived from pMON25496 when compared to the average injury of 6 corn events derived from a construct where the glyphosate tolerance gene expression was driven only by the rice actin promoter and intron (P-Os.Actl/I-Os.Actl). The plants transformed with the double expression cassette possessed a higher level of glyphosate tolerance to vegetative and fertility injury than plants derived from transformation with the single expression cassettes this resulted in improved resistance to yield loss due to glyphosate herbicide application. The pMON25496 construct provides two plant expression cassettes at a single location in nk603 that confers a higher lever of glyphosate tolerance than the triple tandem insertion occurring in the commercial standard, GA21.

Table 1.

Glyphosate Tolerance of nk603 -Vegetative injury, Yield, and Fertility Rating				
Event	Treatment	% Veg injury*	Yield (bushels/acre)	% Yield red.
GA21	Unsprayed	0.3	142.2	
	64 oz Roundup Ultra® at V4 followed by 64 oz at V8	5.3	134.1	5.7
	96 oz Roundup Ultra® at V4 followed by 96 oz at V8	8.3	129.1	9.2
nk603	Unsprayed	0.9	145.6	
	64 oz Roundup Ultra® at V4 followed by 64 oz at V8	2.9	138.5	4.9
	96 oz Roundup Ultra® at V4 followed by 96 oz at V8	4.7	140.1	3.8

	Treatment	Fertility Rating**
nk603	64 oz Roundup® Ultra at V8	4.5
CaMV.35S ev 1	64 oz Roundup® Ultra at V8	2.0
CaMV.35S ev 2	64 oz Roundup® Ultra at V8	2.2
CaMV.35S ev 3	64 oz Roundup® Ultra at V8	2.4

	Treatment	ave. % Veg injury*
nk603 plus 3 additional pMON25496 events	128 oz Roundup® Ultra at V4 followed by 128 oz at V8	22.9
Six P-Os.Actl single cassette events	128 oz Roundup® Ultra at V4 followed by 128 oz at V8	28.9

* Veg injury is observed 10 days after V8 treatment is one measure taken to assess vegetative injury in response to glyphosate treatment. ** Male fertility rating: 4-5 = fully fertile; 3 = significantly reduced pollen shed; 0-2 = completely sterile - highly sterile, not suitable for commercial use.

EXAMPLE 3

[0047] The corresponding flanking DNA molecule from nk603 was cloned by using ligated adapters and nested PCR as described in the Genome Walkers kit (catalog # K1807-1, CloneTech Laboratories, Inc, Palo Alto, CA). First, genomic DNA from the nk603 event was purified by the CTAB purification method (Rogers *et al.*, Plant Mol. Biol. 5:69-76, 1985). The genomic DNA libraries for amplification were prepared according to manufacturer's instructions (Genome Walker™, CloneTech Laboratories, Inc, Palo Alto, CA). In separate reactions, genomic DNA was digested overnight at 37°C with the following blunt-end restriction endonucleases: *Eco*RV, *Sca*I, *Dra*I, *Pvu*II, and *Stu*I (CloneTech Laboratories, Inc, Palo Alto, CA). The reaction mixtures were extracted with phenol:chloroform, the DNA was precipitated by the

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addition of ethanol to the aqueous phase, pelleted by centrifugation, then resuspended in Tris-EDTA buffer (10mM Tris-.HCl, pH 8.0, 1mM EDTA). The purified blunt-ended genomic DNA fragments were ligated to the Genome Walker™ adapters according to the manufacturer's protocol. After ligation, each reaction was heat treated (70°C for 5 min) to terminate the reaction and then diluted 10-fold in Tris-EDTA buffer. One µl of each respective ligation was then amplified in a 50 µl reaction that included 1 µl of respective adapter-ligated library, 1 µl of 10 µM Genome Walker™ adapter primer AP1 (5'GTATATCGACTCACTATAGGGC 3', SEQ ID NO:1), 1 µl of 10 µM nk603 transgene-specific oligonucleotide

(5' TGACGTATCAAAGTACCGACAAAAACATCC 3' SEQ ID NO:2), 1 µl 10 mM deoxyribonucleotides, 2.5 µl dimethyl sulfoxide, 5 µl of 10X PCR buffer containing $MgCl_2$, 0.5 µl (2.5 units)of Amplitaq thermostable DNA polymerase (PE Applied Biosystems, Foster City, CA), and H_2O to 50 µl. The reactions were performed in a thermocycler using calculated temperature control and the following cycling conditions: 1 cycle of 95°C for 9 min; 7 cycles of 94°C for 2 s, 70°C for 3 min; 36 cycles of 94°C for 2 s, 65°C for 3 min; 1 cycle of 65°C for 4 min. One µl of each primary reaction was diluted 50-fold with water and amplified in a secondary reaction (1 µl of respective diluted primary reaction, 1 µl of 10 µM Genome Walker™ nested adapter primer AP2 (5'ACTATAGGGCACGCGTGGT 3', SEQ ID NO:3, supplied by manufacturer), 1 µl of 10 µM nk603 transgene-specific nested oligonucleotide

(5' CTTTGTTTTATTTTGGACTATCCCGACTC 3', SEQ ID NO:4), 1 µl 10 mM deoxyribonucleotides, 2.5 µl dimethyl sulfoxide, 5 µl of 10X PCR buffer containing $MgCl_2$, 0.5 µl (2.5 units)of Amplitaq thermostable DNA polymerase (PE Applied Biosystems, Foster City, CA), and H_2O to 50 µl] using the following cycling conditions: 1 cycle of 95°C for 9 min; 5 cycles of 94°C for 2 s, 70°C for 3 min; 24 cycles of 94°C for 2 s, 65°C for 3 min; 1 cycle of 65° C for 4 min.

[0048] PCR products, representing 5' regions that span the junction between the nk603 transgenic insertion and the neighboring flanking corn DNA were purified by agarose gel electrophoresis followed by purification from the agarose matrix using the QIAquick Gel Extraction Kit (catalog # 28704, Qiagen Inc., Valencia, CA) and direct cloning into the pGEM-T Easy vector (catalog. # A1360, Promega, Madison, WI). The identity of the cloned PCR products and relationship to the Mlu I fragment of pMON25496 was confirmed by DNA sequence analysis (ABI Prism™ 377, PE Biosystems, Foster City, CA and DNASTAR sequence analysis software, DNASTAR Inc., Madison, WI).

[0049] Similarly, the nk603 3' flanking corn DNA sequence was amplified and cloned using nested gene specific primers, such as, SEQ ID NO:5

(5' AGATTGAATCCTGTTGCCGGTCTTGC 3') and SEQ ID NO:6

(5' GCGGTGTCATCTATGTTACTAGATCGGG 3') that anneal to the T-AGRTU.nos transcriptional terminator. Two T-AGRTU.nos transcriptional terminators are present in the nk603 transgenic/genomic insertion, one internal in the construct and one at the 3' end of the construct adjacent to corn genomic DNA. The PCR products produced in this reaction were sequenced and the DNA sequence that spans the junction between transgene and flanking sequence was distinguished from products of the internal T-AGRTU.nos by comparison to the known genetic element sequences of the pMON25496 construct as previously described.

[0050] Corn DNA sequence flanking both sides of the transgenic insertion was determined for nk603 by sequencing the Genome Walker™ -derived amplification products and alignment to known transgene sequence. At the 5' end of the transgenic insertion, the sequence of a 498 bp segment around the insertion junction was determined (SEQ ID NO:7). This consisted of 304 base pairs (bp) of the flanking maize genomic DNA sequence (nucleotides 1-304 of SEQ ID NO:7), 45 bp of pMON25496 construct DNA sequence (nucleotides 305-349 of SEQ ID NO:7) and 149 bp of DNA sequence from the 5' end of P-Os.Act1 (nucleotides 350-498 of SEQ ID NO:7).

[0051] The DNA sequence was determined for a 1183 bp segment around the 3' insertion junction (SEQ ID NO:8), that begins with 164 bp of the T-AGRTU.nos transcriptional terminator (nucleotides 1-164 of SEQ ID NO:8), 217 bp of pMON25496 construct DNA sequence (nucleotides 165-381 of SEQ ID NO:8), 305 bp of the maize plastid genes, *rps11* and *rpoA* (partial segments of each gene corresponding to bases 63-363 of Genbank accession X07810, corresponding to bases 382-686 of SEQ ID NO:8), and the remaining DNA sequence consisting of maize genomic DNA sequence flanking the integration site (corresponding to bases 687-1183 of SEQ ID NO:8).

[0052] The junction DNA molecules, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12 are novel DNA molecules in nk603 and are diagnostic for corn plant nk603 and its progeny. The junction molecules in SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11 represent about 9 nucleotides on each side of an insertion site of a transgene DNA fragment and corn genomic DNA. SEQ ID NO:9 is found at nucleotide positions 296-314 of SEQ ID NO:7. The junction sequences SEQ ID NO:10 and 11 are located at nucleotide positions 373-390 and 678-695, respectively, of SEQ ID NO:8, representing a junction DNA molecule of construct DNA sequence with corn plastid DNA sequence (SEQ ID NO:10) and construct sequence with corn genomic DNA sequence (SEQ ID NO:11). SEQ ID NO:12 is located at nucleotide position 156-173 of SEQ ID NO:8 and represents a novel DNA molecule in nk603 due to it being a fusion of the T-AGRTU.nos terminator sequence with an inverted fragment of the rice actin promoter DNA sequence.

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EXAMPLE 4

[0053] DNA event primer pairs are used to produce an amplicon diagnostic for nk603. These event primer pairs include but are not limited to SEQ ID NO:13 and SEQ ID NO:14 for the 5' amplicon DNA molecule and SEQ ID NO: 15 and SEQ ID NO:16 for the 3' amplicon DNA molecule. In addition to these primer pairs, any primer pair derived from SEQ ID NO:7 and SEQ ID NO:8 that when used in a DNA amplification reaction produces a DNA amplicon diagnostic for nk603 is an aspect of the present invention. The amplification conditions for this analysis is illustrated in Table 2 and Table 3 for the 5' transgene insert/genomic junction region. The same method is applied for amplification of the 3' amplicon DNA molecule using primer DNA molecules SEQ ID NO:15 and SEQ ID NO:16, however, any modification of these methods that use DNA molecules or complements thereof to produce an amplicon DNA molecule diagnostic for nk603 is within the ordinary skill of the art. In addition, a control primer pair (SEQ ID NO:17 and 18) for amplification of an endogenous corn gene is included as an internal standard for the reaction conditions. The analysis of nk603 plant tissue DNA extract sample should include a positive tissue DNA extract control from nk603, a negative DNA extract control from a corn plant that is not nk603, and a negative control that contains no template corn DNA extract. Additional DNA primer molecules of sufficient length can be selected from SEQ ID NO:7 and SEQ ID NO:8 by those skilled in the art of DNA amplification methods, and conditions optimized for the production of an amplicon that may differ from the methods shown in Table 2 and Table 3 but result in an amplicon diagnostic for nk603. The use of these DNA primer sequences with modifications to the methods of Table 2 and 3 are within the scope of the invention. The amplicon wherein at least one DNA primer molecule of sufficient length derived from SEQ ID NO:7 and SEQ ID NO:8 that is diagnostic for nk603 is an aspect of the invention. The amplicon wherein at least one DNA primer of sufficient length derived from any of the genetic elements of pMON25496 that is diagnostic for nk603 is an aspect of the invention. The assay for the nk603 amplicon can be performed by using a Stratagene Robocycler, MJ Engine, Perkin-Elmer 9700, or Eppendorf Mastercycler Gradient thermocycler as shown in Table 3, or by methods and apparatus known to those skilled in the art

Table 2.

PCR procedure and reaction mixture for the confirmation of nk603 5' transgene insert/genomic junction region.			
Step	**Reagent**	**Amount**	**Comments**
1	Nuclease-free water	add to final volume of 20 µl	-
2	10X reaction buffer (with $MgCl_2$)	2.0 µl	1X final concentration of buffer, 1.5 mM final concentration of $MgCl_2$
3	10 mM solution of dATP, dCTP, dGTP, and dTTP	0.4 µl	200 µM final concentration of each dNTP
4	event primer (SEQ ID NO:13) (resuspended in 1X TE buffer or nuclease-free water to a concentration of 10 µM)	0.4 µl	0.2 µM final concentration
5	event primer (SEQ ID NO:14) (resuspended in 1X TE buffer or nuclease-free water to a concentration of 10 µM)	0.4 µl	0.2 µM final concentration
6	control primer (SEQ ID NO: 17) (resuspended in 1X TE buffer or nuclease-free water to a concentration of 10 µM)	0.2 µl	0.1 µM final concentration
7	control primer (SEQ ID NO:18) (resuspended in 1X TE buffer or nuclease-free water to a concentration of 10 µM)	0.2 µl	0.1 µM final concentration
8	RNase, DNase free (500 ng/µl)	0.1 µl	50 ng/reaction
9	*REDTaq* DNA polymerase (1 unit/µl)	1.0 µl (recommended to switch pipets prior to next step)	1 unit/reaction

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Table 2. (continued)

PCR procedure and reaction mixture for the confirmation of nk603 5' transgene insert/genomic junction region.			
Step	**Reagent**	**Amount**	**Comments**
10	Extracted DNA (template): • Samples to be analyzed * individual leaves * pooled leaves (maximum of 50 leaves/pool) • Negative control • Negative control • Positive control	 • 10-200 ng of genomic DNA • 200 ng of genomic DNA • 50 ng of corn genomic DNA (notnk603) • no template DNA • 50 ng of nk603 genomic DNA	-

Table 3. Suggested PCR parameters for different thermocyclers

Gently mix and, if needed (no hot top on thermocycler), add 1-2 drops of mineral oil on top of each reaction. Proceed with the PCR in a Stratagene Robocycler, MJ Engine, Perkin-Elmer 9700, or Eppendorf Mastercycler Gradient thermocycler using the following cycling parameters.

Note: The MJ Engine or Eppendorf Mastercycler Gradient thermocycler should be run in the calculated mode. Run the Perkin-Elmer 9700 thermocycler with the ramp speed set at maximum.

Cycle No.	Settings: Stratagene Robocycler
1	94°C 3 minutes
38	94°C 1 minute 60°C 1 minute 72°C 1 minute and 30 seconds
1	72°C 10 minutes

Cycle No.	Settings: MJ Engine or Perkin-Elmer 9700
1	94°C 3 minutes
38	94°C 10 seconds 60°C 30 seconds 72°C 1 minute
1	72°C 10 minutes

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Cycle No.	Settings: Eppendorf Mastercycler Gradient
1	94°C 3 minutes
38	94°C 15 seconds 60°C 15 seconds 72°C 1 minute
1	72°C 10 minutes

[0054] A deposit of the Monsanto Company, corn seed of event PV-ZMGT32(nk603) disclosed above has been made under the Budapest Treaty with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110. The ATCC accession number is PTA-2478. The deposit will be maintained in the depository for a period of 30 years, or 5 years after the last request, or for the effective life of the patent, whichever is longer, and will be replaced as necessary during that period.

[0055] Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

[0056] All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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Bayer EX1008 (Part 8 of 9)

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SEQUENCE LISTING

<110> Monsanto Co
Behr, Carl
Hironaka, Catherine
Heck, Gregory
You, Jinsong

<120> Corn Event PV-ZMGT32(nk603) and Composition and Methods for Detection Thereof

<130> 38-21(52258)B

<150> 60/213,567
<151> 2000-06-22

<150> 60/241,215
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Bayer EX1008 (Part 8 of 9)

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Bayer EX1008 (Part 8 of 9)

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Bayer EX1008 (Part 8 of 9)

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Claims

1. A DNA construct comprising: a first and a second expression cassette, wherein said first expression cassette in operable linkage comprises (i) a rice actin 1 promoter; (ii) a rice actin 1 intron; (iii) a chloroplast transit peptide encoding DNA molecule; (iv) a glyphosate tolerant EPSPS encoding DNA molecule; and (v) a transcriptional terminator DNA molecule; and said second expression cassette comprising in operable linkage (a) a CaMV 35S promoter; (b) a Hsp70 intron; (c) a chloroplast transit peptide encoding DNA molecule; (d) a glyphosate tolerant EPSPS encoding DNA molecule; and (e) a transcriptional terminator DNA molecule.

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2. A DNA construct of claim 1, wherein the glyphosate tolerant EPSPS encoding DNA molecule consists of an AGRTU. *aroA*:CP4 DNA molecule.

3. A DNA molecule comprising a nucleotide sequence identified as SEQ ID NO:7 or SEQ ID NO:8.

4. A pair of DNA molecules comprising: a first DNA molecule and a second DNA molecule, wherein the DNA molecules are of sufficient length of contiguous nucleotides of SEQ ID NO:7 or its complement to function as DNA primers or probes diagnostic for DNA extracted from corn plant PV-ZMGT32(nk603) or progeny thereof.

5. A pair of DNA molecules comprising: a first DNA molecule and a second DNA molecule, wherein the DNA molecules are of sufficient length of contiguous nucleotides of SEQ ID NO:8 or its complement to function as DNA primers or probes diagnostic for DNA extracted from corn plant PV-ZMGT32(nk603) or progeny thereof.

6. A method of detecting the presence of a DNA molecule selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8 in a DNA sample, the method comprising:

(a) extracting a DNA sample from a corn plant;
(b) contacting the DNA sample with a DNA primer pair comprising DNA primer molecules of sufficient length of contiguous nucleotides of SEQ ID NO:7 or its complement, or SEQ ID NO:8 or its complement;
(c) providing a nucleic acid amplification reaction condition;
(d) performing said nucleic acid amplification reaction, thereby producing a DNA amplicon molecule; and
(e) detecting the DNA amplicon molecule.

7. A method of detecting the presence of a DNA molecule selected from the group consisting of SEQ ID NO:7 and SEQ ID NO:8 in a DNA sample, the method comprising:

(a) extracting a DNA sample from a corn plant;
(b) contacting the DNA sample with a DNA molecule identified as SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO: 11, or SEQ ID NO:12, wherein said DNA molecule is a DNA probe that hybridizes under stringent hybridization conditions with the DNA molecule selected from the group consisting of SEQ ID NO:7 or SEQ ID NO:8, and does not hybridize under the stringent hybridization conditions with a DNA sample not containing the DNA molecule identified as SEQ ID NO:7 or SEQ ID NO:8;
(c) subjecting the sample and probe to stringent hybridization conditions; and detecting hybridization of the probe to the DNA.

8. A DNA molecule selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO: 12, and complements thereof.

9. A method of breeding a glyphosate tolerant trait into corn plants comprising

a) extracting a DNA sample from progeny corn plants;
b) contacting the DNA sample with a marker nucleic acid molecule selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, or complements thereof.
c) performing a marker assisted breeding method for the glyphosate tolerant trait, wherein the glyphosate tolerant trait is genetically linked to a complement of the marker nucleic acid molecule;

10. A method of producing a corn plant that tolerates application of glyphosate herbicide comprising:

(a) transforming a corn cell with the DNA construct of claim 1;
(b) selecting said corn cell for tolerance to application of glyphosate;
(c) growing said corn cell into a fertile corn plant.

11. A DNA detection kit comprising: at least one DNA molecule of sufficient length of contiguous nucleotides homologous or complementary to SEQ ID NO:7 or SEQ ID NO:8 that functions as a DNA primer or probe specific for corn event PV-ZMGT32(nk603) and/or its progeny.

12. A glyphosate tolerant corn plant, its progeny, or its parts thereof, said corn plant provided by ATCC seed deposit PTA-2478.

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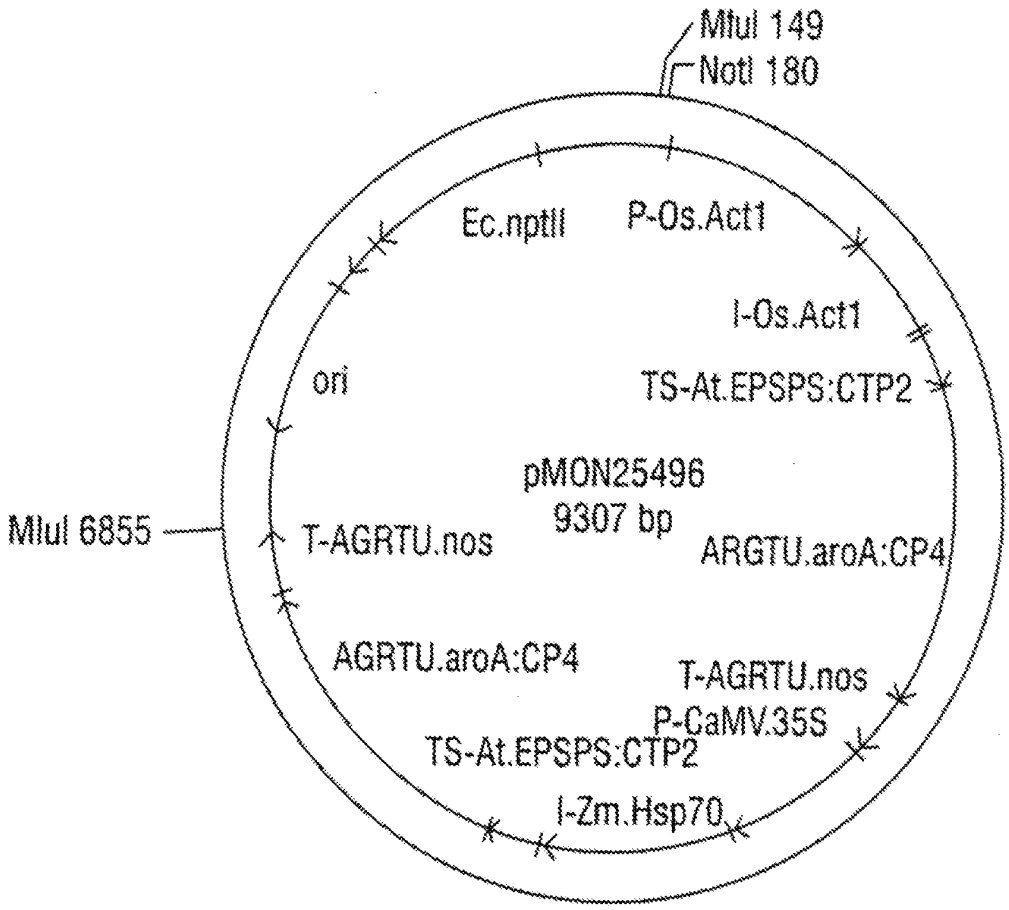


FIG. 1

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European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 01 20 2314

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	US 5 948 956 A (BERG JOHN BRADLEY ET AL) 7 September 1999 (1999-09-07) * column 10, line 11 - line 26; figure 4 * * column 10, line 55 - column 12, line 5 * ---	1,2,10	C12N15/82 A01H5/00 A01H5/10
Y,D	WINDELS P ET AL: "Development of a line specific GMO detection method: A case study." MEDEDELINGEN FACULTEIT LANDBOUWKUNDIGE EN TOEGEPASTE BIOLOGISCHE, vol. 64, no. 5b, 1999, pages 459-462, XP001032975 Thirteenth Forum for Applied Biotechnology.;Gent, Belgium; September 22-23, 1999, 1999 * the whole document * ---	3-8,11	
Y,D	WO 98 44140 A (DEKALB GENETICS CORP) 8 October 1998 (1998-10-08) * page 50, line 21 - line 26 * * page 63, line 20 - line 23 *	3-8,11	
A	* page 41, line 17 - page 46, line 20 * -----	1,2,10	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12N

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

12

Reason for the limitation of the search:

Article 53 (b) EPC - Plant variety

Place of search	Date of completion of the search	Examiner
BERLIN	16 October 2001	De Kok, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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European Patent Office

Application Number
EP 01 20 2314

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☒ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

☐ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

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European Patent Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 01 20 2314

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1, 2, 10

A DNA construct comprising a first and second expression cassette, wherein the first expression cassette comprises a glyphosate tolerant EPSPS under the control of a rice actin-1 promoter and the second expression cassette comprises a glyphosate tolerant EPSPS under the control of a CaMV 35S promoter

2. Claims: 3-9 11 12

A glyphosate tolerant corn plant, PV-ZMGT32(nk206), ATCC seed deposit PTA-2478 and DNA molecules for the specific detection of said plant.

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Bayer EX1008 (Part 8 of 9)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 01 20 2314

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

16-10-2001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5948956 A	07-09-1999	AU 9806698 A	03-05-1999
		WO 9919499 A1	22-04-1999
WO 9844140 A	08-10-1998	US 6040497 A	21-03-2000
		AU 6882298 A	22-10-1998
		BR 9808475 A	23-05-2000
		EP 0975778 A1	02-02-2000
		WO 9844140 A1	08-10-1998

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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PCT

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/82, 15/10 — A3

(11) International Publication Number: WO 00/09727

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/US99/18394

(22) International Filing Date: 12 August 1999 (12.08.99)

(30) Priority Data:

60/096,288	12 August 1998 (12.08.98)	US
60/111,146	7 December 1998 (07.12.98)	US
60/112,746	17 December 1998 (17.12.98)	US

(71) Applicant: MAXYGEN, INC. [US/US]; 515 Galveston Drive, Redwood City, CA 94063 (US).

(72) Inventors: SUBRAMANIAN, Venkitswaran; 3980 Corte Mar De Hierba, San Diego, CA 94063 (US). STEMMER, Willem, P., C.; 108 Kathy Court, Los Gatos, CA 95030 (US). CASTLE, Linda, A.; 784 Hans Avenue, Mountain View, CA 94040 (US). MUCHHAL, Umesh, S.; Apartment 189, 1600 Villa Street, Mountain View, CA 94041 (US). SIEHL, Daniel, L.; 310 Durham Street, Menlo Park, CA 94025 (US).

(74) Agents: PETITHORY, Joanne, R.; Maxygen, Inc., 515 Galveston Drive, Redwood City, CA 94063 (US) et al.

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

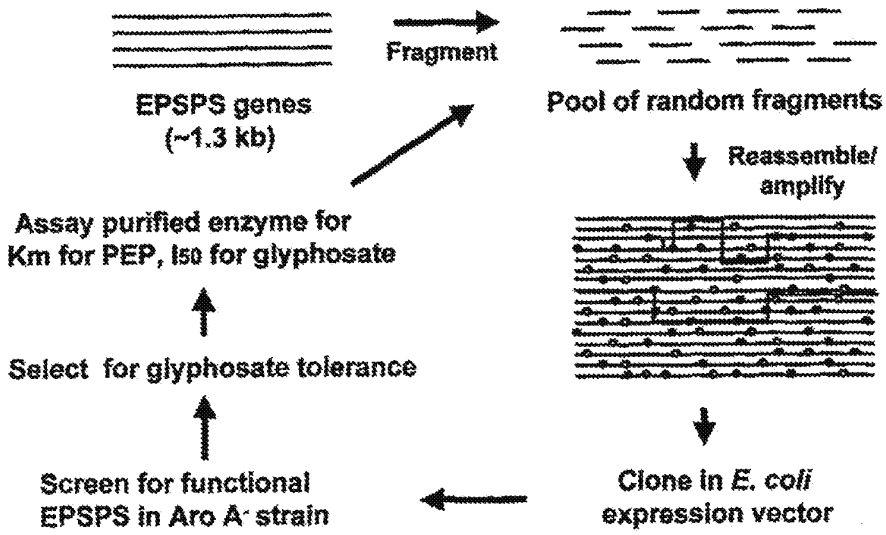
Published

With a revised version of the international search report.

(88) Date of publication of the international search report: 18 May 2000 (18.05.00)

(88) Date of publication of the revised version of the international search report: 6 July 2000 (06.07.00)

(54) Title: DNA SHUFFLING TO PRODUCE HERBICIDE SELECTIVE CROPS



(57) Abstract

Methods of shuffling DNA to obtain recombinant herbicide tolerance nucleic acids encoding proteins having new or improved herbicide tolerance activities, libraries of shuffled herbicide tolerance nucleic acids, transgenic plants and DNA shuffling mixtures are provided.

*(Referred to in PCT Gazette No. 27/2000, Section II)

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Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
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BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
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CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon			PT	Portugal		
CN	China	KR	Republic of Korea	RO	Romania		
CU	Cuba	KZ	Kazakstan	RU	Russian Federation		
CZ	Czech Republic	LC	Saint Lucia	SD	Sudan		
DE	Germany	LI	Liechtenstein	SE	Sweden		
DK	Denmark	LK	Sri Lanka	SG	Singapore		
EE	Estonia	LR	Liberia				

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/18394

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/10

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 31837 A (DELCARDAYRE STEPHEN B ;MAXYGEN INC (US); MINSHULL JEREMY (US); NES) 23 July 1998 (1998-07-23) page 28, line 19 -page 31, line 37 ---	1-60
X	WO 98 31816 A (MINSHULL JEREMY S ;SOUZA MERVYN L DE (US); UNIV MINNESOTA (US); SA) 23 July 1998 (1998-07-23) the whole document ---	1-3,28, 30,35,37
X	MOURAD, G., ET AL.: "Intragenic recombination in the CSR1 locus of Arabidopsis" MOL. GEN. GENET., vol. 243, 1994, pages 178-184, XP002128867 the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
4 April 2000	07.04.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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International Application No
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[19] 中华人民共和国国家知识产权局

[51] Int. Cl[7]
C07D487/04
C07D333/34 C07D333/38
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[71] 申请人 美国陶氏益农公司
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代理人 程 伟

权利要求书 3 页 说明书 30 页

[54] 发明名称 用作除草剂的 N-((1,2,4,)三唑并噻基)噻吩磺酰胺化合物

[57] 摘要

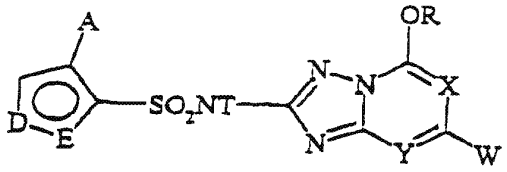
N-(三唑并噻基)噻吩磺酰胺化合物是由适当取代的 2-氨基[1, 2, 4]三唑并[1, 5-c]嘧啶, 2-氨基[1, 2, 4]三唑并[1, 5-a]嘧啶和 2-氨基[1, 2, 4]三唑并[1, 5-a]吡啶化合物与适当取代的噻吩磺酰氯化合物制备的。已发现上述化合物可用作除草剂。

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1. 一种式(I)所示的 N-(三唑并嘧基)噻吩磺酰胺化合物:



(I)

其中

X 代表 CH 或 N;

Y 代表 CZ 或 N,条件是 X 和 Y 不同时为 N;

W 代表 H 或 OR,条件当 Y 是 CZ 时,则 W 是 H;

Z 代表 R,OR 或卤素;

D 和 E 代表 S 或 CB,条件是 D 或 E 其中之一是 S;

A 和 B 各自独立地代表 H,卤素,CF_3,R,OR'或 CO_2R";

T 代表 H, SO_2R",C(O)R", C(O)OR",C(O)$NR"_2$,或 $CH_2CH_2C(O)OR$";

R 代表 CH_3 或 CH_2CH_3;

R' 代表 C_1-C_4 烷基,C_3-C_4 烯基,或 C_3-C_4 炔基,它们各自任选具有至多两个氯,溴或 O(C_1-C_4)烷基取代基或至多最多可能数量的氟取代基;

R" 代表 H 或 C_1-C_4 烷基;

以及,当 T 代表 H 时,它们的农业上可接受的盐。

2. 如权利要求 1 所述的化合物,其中 T 代表 H 或其农业上可接受的盐。

3. 如权利要求 1 或 2 所述的化合物,其中 X 代表 N,Y 代表 CZ 以及 W 代表 H。

4. 如权利要求 1 或 2 所述的化合物,其中 X 代表 CH 以及 Y 代表 N。

5. 如权利要求 1 或 2 所述的化合物,其中 X 代表 CH,Y 代表 CZ 以及 W 代表 H。

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6. 如上述权利要求中任一项所述的化合物,其中 D 代表 S 以及 E 代表 CB。

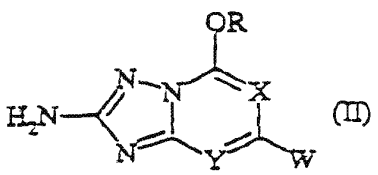
7. 如上述权利要求中任一项所述的化合物,其中 B 代表 CO_2R''。

8. 如上述权利要求中任一项所述的化合物,其中 A 代表 OR'或 CO_2R''。

9. 一种组合物,含有除草量的如权利要求 1-8 中任一项所述的 N-(三唑并嘧基)噻吩磺酰胺化合物以及混合农业上可接受的助剂或载体。

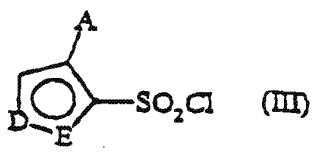
10. 一种防治不需要植物的方法,该方法包括将除草有效量的如权利要求 1-8 中任一项所述的 N-(三唑并嘧基)噻吩磺酰胺化合物施用至上述植物或它们的生长区。

11. 如权利要求 1 所述的N-(三唑并嘧基)噻吩磺酰胺化合物的制备方法,该方法包括将式(II)化合物:



(II)

与式 III 的噻吩磺酰氯化合物接触



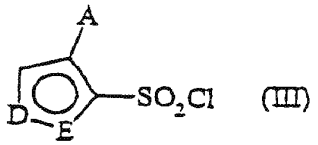
(III)

其中 A,D,E,R,W,X 和 Y 如式 I 化合物中所述定义

该接触是在一种极性、非质子溶剂的无水条件下并加入吡啶作为碱以及催化量的二甲基亚砜的条件下进行。

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12. 一种式 III 的噻吩磺酰氯化合物:



(III)

其中

D 和 E 代表 S 或 CB,条件是 D 或 E 之一是 S;

A 和 B 各自独立地代表 H,卤素,CF_3,R,OR'或 CO_2R",条件是 A 或 B 中至少之一代表 OR'或 CO_2R";

R 代表 CH_3 或 CH_2CH_3;

R' 代表 C_1-C_4 烷基,C_3-C_4 烯基,或 C_3-C_4 炔基,它们各自任选具有至多两个氯,溴或 O(C_1-C_4)烷基取代基或至多最多可能数量的氟取代基;以及

R" 代表 H 或 C_1-C_4 烷基

条件是当 D 代表 S 以及 A 代表 H 时,则 B 不能代表 CO_2R";当 D 代表 S 以及 B 代表 H 时,则 A 不能代表 CO_2R";当 E 代表 S 以及 B 代表 H 时,则 A 不能代表 CO_2R";或当 E 代表 S 以及 B 代表 H 时,则 A 不能代表 OCH_3。

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用作除草剂的 N-((1,2,4)三唑并嗪基)噻吩磺酰胺化合物

本发明涉及取代的噻吩磺酰胺化合物,含有该化合物的除草组合物,以及该化合物用于防治不需要植物的用途。

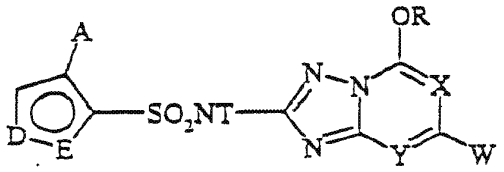
通过化学制剂即除草剂对不需要植物的防治是现代农业和土地管理中的重要组成部分。虽然已知许多化学品可用于防治不需要植物,然而理想的新化合物通常是具有更高活性,对特定植物种类更有效,对需要的植物更低伤害,对人或环境更安全,更低的使用成本,或具有其它有利特性的化合物。

许多取代的苯磺酰胺化合物是已知的并已知其中的某些化合物具有除草活性。例如,美国专利 4,638,075 中公开了某些 N-([1,2,4]三唑并[1,5-a]嘧啶-2-基)苯磺酰胺化合物以及它们的除草应用,以及美国专利 4,685,958 中公开了某些 N-([1,2,4]三唑并[1,3,5]三嗪-2-基)苯磺酰胺化合物。此外,在美国专利 5,858,924 中公开了某些 N-([1,2,4]三唑并[1,5-c]嘧啶-2-基)苯磺酰胺,N-([1,2,4]三唑并[1,5-c]-嘧啶-2-基)吡啶磺酰胺,N-([1,2,4]三唑并[1,5-a]吡啶-2-基)苯磺酰胺,以及 N-([1,2,4]三唑并[1,5-a]吡啶-2-基)吡啶磺酰胺化合物。还已知某些 N-苯基芳基磺酰胺化合物并已知这些化合物具有除草活性。例如,美国专利 5,163,995 中公开了某些N-(取代苯基)[1,2,4]三唑并[1,5-c]嘧啶-2-磺酰胺化合物以及美国专利 5,571,775 中公开了某些 N-(取代苯基)[1,2,4]三唑并[1,5-a]-吡啶-2-磺酰胺化合物。

现已发现新的一类 N-(三唑并嗪基)噻吩磺酰胺化合物是采用出苗前或出苗后施用方法防治不需要植物的有效除草剂,这些化合物包括 N-([1,2,4]三唑并[1,5-c]嘧啶-2-基)噻吩磺酰胺,N-([1,2,4]三唑并[1,5-a]嘧

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啶-2-基)噻吩磺酰胺,以及 N-([1,2,4]-三唑并[1,5-a]吡啶-2-基)噻吩磺酰胺化合物。本发明涉及式 I 的 N-(三唑并嗪基)噻吩磺酰胺化合物:



(I)

其中

X 代表 CH 或 N;

Y 代表 CZ 或 N,条件是 X 和 Y 不同时为 N;

W 代表 H 或 OR,条件当 Y 是 CZ 时,则 W 是 H;

Z 代表 R,OR 或卤素;

D 和 E 代表 S 或 CB,条件是 D 或 E 其中之一是 S;

A 和 B 各自独立地代表 H,卤素,CF_3,R,OR'或 CO_2R";

T 代表 H,SO_2R",C(O)R",C(O)OR",$C(O)NR"_2$,或 $CH_2CH_2C(O)OR$";

R 代表 CH_3 或 CH_2CH_3;

R' 代表 C_1-C_4 烷基,C_3-C_4 烯基,或 C_3-C_4 炔基,它们各自任选具有至多两个氯,溴或 O(C_1-C_4)烷基取代基或至多可能的最多数量的氟取代基;

R" 代表 H 或 C_1-C_4 烷基;

以及,当 T 代表 H 时,它们的农业上可接受的盐。

本发明优选的化合物是当 D 代表 S 以及 T 代表 H 时,A 代表 OR'或 B 代表 CO_2R"的化合物。

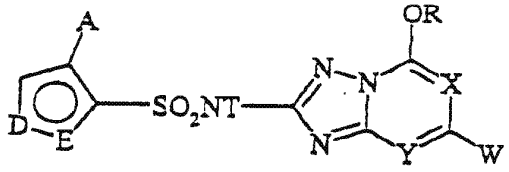
本发明还涉及含有除草量的式 I 化合物与一种或多种农业上可接受的助剂或载体混合的组合物以及式 I 化合物作为除草剂的用途。通常优选应用适合的本发明化合物获得灭生性的植物防治。它们可以防治禾本科和阔叶杂草。通常优选将上述化合物苗后施用至不需要的植物。

通常将本发明的 N-(三唑并嗪基)噻吩磺酰胺化合物描述为取代的 N-([1,2,4]三唑并[1,5-c]嘧啶-2-基)噻吩磺酰胺,N-([1,2,4]三唑并[1,5-a]嘧啶-2-基)噻吩磺酰胺,以及 N-([1,2,4]-三唑并[1,5-a]吡啶-2-基)噻吩磺酰胺化合物。它们的特征表现为取代的噻吩磺酰胺化合物在酰胺氮原子上具有取代的[1,2,4]三唑并[1,5-c]嘧啶-2-基,取代的[1,2,4]三唑并[1,5-a]

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嘧啶-2-基或取代的[1,2,4]三唑并[1,5-a]吡啶-2-基部分。

本发明的除草化合物是通式 I 的 N-(三唑并嗪基)噻吩磺酰胺化合物:



(I)

其中 X 代表 N 的上述化合物含有取代的 N-([1,2,4]三唑并[1,5-c]嘧啶-2-基)部分,其中 Y 代表 N 的那些化合物含有取代的 N-([1,2,4]三唑并[1,5-a]嘧啶-2-基)部分以及其中 X 代表 C-H 和 Y 代表 C-Z 的那些化合物含有取代的 N-([1,2,4]-三唑并[1,5-a]吡啶-2-基)部分。其中 E 代表 S 的化合物是 2-噻吩磺酰胺化合物以及其中 D 代表 S 的化合物是 3-噻吩磺酰胺化合物。本发明化合物的另一特征是在三唑并嗪环的六元环部分的桥头氮邻位处具有甲氧基或乙氧基取代基以及噻吩环上与磺酰胺邻位处具有至少一个取代基(A)。

本发明化合物包括其中 X 是 N 或 CH 的式 I 化合物。通常优选其中 X 是 N 的化合物。然而,某些情况下优选其中 X 是 CH 的化合物。

本发明化合物包括其中 Y 是 N,条件是 X 不是 N;或 Y 是 CZ,其中 Z 是甲基,乙基,甲氧基,乙氧基或卤素的式 I 化合物。通常优选 Y 是 CZ 的化合物。然而,某些情况下优选 Y 是 N 的化合物。通常优选 Z 是甲氧基的化合物。

本发明化合物包括其中 D 和 E 代表 S 或 CB 的式 I 化合物,条件是 D 或 E 中的一个以及仅有一个是 S。通常优选其中 D 代表 S 的噻吩-3-磺酰胺。

本发明化合物包括其中 A 和 B 各自独立地代表 H,卤素,CF_3,R,OR'或 CO_2R"的式 I 化合物。A 优选 OR'或 CO_2R",以及最优选 OR'。

本发明化合物包括其中 W 代表 H 或 OR,条件是当 Y 是 CZ 时,W 是 H 的式 I 化合物。当 Y 是 N 时,W 优选是甲氧基。

本发明化合物中,R 可以是 CH_3 或 CH_2CH_3。OR 中,R 优选是 CH_3。

本发明化合物中,R'可以是 C_1-C_4烷基,C_3-C_4烯基,或 C_3-C_4炔基,它们

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各自任选具有至多两个氯,溴或 O(C_1-C_4)烷基取代基或至多可能的最多数量的氟取代基。对于 OR',R'优选是 C_1-C_4 烷基,其任选具有至多两个氯,溴或 O(C_1-C_4)烷基取代基或至多可能的最多数量的氟取代基。

式 I 化合物包括其中 T 代表氢,烷基磺酰基(SO_2R''),酰基(C(O)R''),烷氧基羰基(C(O)OR''),氨基羰基(C(O)NR''_2),或 2-(烷氧基羰基)乙基($CH_2CH_2C(O)OR''$)的那些化合物。优选其中 T 代表氢的上述化合物。本发明还包括其中 T 代表氢的式 I 化合物的农业上可接受的盐。

本发明化合物中,R''可以是 H 或 C_1-C_4 烷基。R''优选是 CH_3 或 CH_2CH_3。

另外,将具有各自可能的优选,更优选,最优选,适合的,以及特别有利的取代基互相之间组合的式 I 化合物也看作是本发明的重要实施方案。

本发明中使用的术语烷基,烯基以及炔基(包括在卤代烷基和烷氧基中被修饰的)包括直链,支链以及环状基团。因此,代表性的烷基是甲基,乙基,1-甲基乙基,丙基,1,1-二甲基乙基以及环丙基。通常优选甲基和乙基。某些情况下本发明的烷基是指正(n),异(i),仲(s)或叔(t)烷基。带有至多最多可能数量的氟取代基的代表性的烷基包括三氟甲基,一氟甲基,2,2,2-三氟乙基,2,3-二氟丙基等;通常优选三氟甲基。术语卤素包括氟,氯,溴,和碘。

本发明使用的术语“农业上可接受的盐”表示式 I 化合物的酸性磺酰胺质子被阳离子替换的化合物,其中阳离子本身对将要处理的作物既没有除草活性,同时也对施药人员,环境,或将要处理的任何作物的最终使用者没有明显的药害的阳离子。适合的阳离子包括,例如,由碱金属或碱土金属衍生的阳离子以及由氨和胺衍生的阳离子。优选的阳离子包括钠,钾,镁,以及下式的铵阳离子:

$$R^2R^3R^4NH^+$$

其中 R^2,R^3 和 R^4 各自独立地代表氢或(C_1-C_{12})烷基,(C_3-C_{12})环烷基,或(C_3-C_{12})烯基,上述基团各自任选被一个或多个羟基,(C_1-C_8)烷氧基,(C_1-C_8)烷硫基或苯基取代;条件是 R^2,R^3,和 R^4 在空间上是相容的。此外,R^2,R^3,和 R^4 中的任意两个基团一起可代表含有 1-12 个碳原子以及至多两个氧或硫原子的脂族双官能部分。式 I 化合物的盐的制备可通过将其中 V 代表氢的式 I 化合物用金属氢氧化物,如氢氧化钠,氢氧化钾或氢

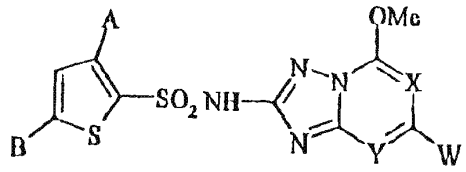
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氧化镁,或胺,如氨,三甲基胺,羟乙基胺,二烯丙基胺,2-丁氧基乙胺,吗啉,环十二胺或苄胺处理获得。

表 1 和 2 的化合物是本发明化合物的实例。一些特别优选的式 I 化合物,可根据需要防治的杂草种类、存在的作物(如果有的话)、及其它因素而确定,包括表 1 和 2 中的下述化合物:N-(5,8-二甲氧基[1,2,4]三唑并[1,5-c]嘧啶-2-基)-4-甲氧基噻吩-3-磺酰胺和 N-(5,8-二甲氧基[1,2,4]三唑并[1,5-c]嘧啶-2-基)-2-羧甲基-噻吩-3-磺酰胺。

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表 1　磺酰胺化合物

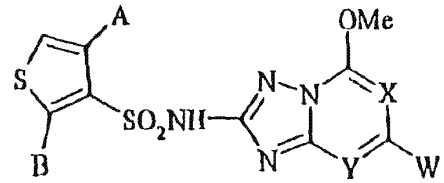


化合物序号	A	B	X	Y	W	形状	熔点 ℃	元素分析计算值/实测值		
								%C	%H	%N
1	OCH_3	H	N	C- OCH_3	H	棕褐色粉末	227-228	38.81 38.32	3.53 3.49	18.86 18.91
2	OCH_3	H	N	C-Cl	H	粉红色粉末	226-227	35.16 35.50	2.68 2.83	18.64 18.30
3	OCH_3	Cl	N	C- OCH_3	H	白色粉末	221-222	35.52 35.65	2.98 2.94	17.26 16.99
4	OCH_3	Cl	CH	N	OCH_3	白色粉末	218-219	35.52 35.44	2.98 2.89	17.26 16.91
5	OCH_3	CF_3	CH	N	OCH_3	棕褐色粉末	218-219	35.54 35.77	2.75 2.76	15.94 15.51

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表 2　磺酰胺化合物



化合物序号	A	B	X	Y	W	形状	熔点 ℃	元素分析计算值/实测值		
								%C	%H	%N
6	H	CO_2CH_3	N	C- OCH_3	H	棕褐色粉末	277-279	39.09 38.72	3.38 3.13	17.53 17.09
7	OCH_3	H	N	C- OCH_3	H	白色粉末	236-237	38.81 39.05	3.53 3.54	18.86 18.55
8	H	OCH_3	N	C- OCH_3	H	灰色粉末	220-221	38.81 38.83	3.53 3.45	18.86 18.66
9	H	OCH_3	N	C- CH_3	H	白色粉末	220-221	40.56 40.59	3.69 3.60	19.71 19.65
10	OCH_3	CF_3	N	C- OCH_3	H	棕褐色粉末	231-232	35.54 35.28	2.75 2.71	15.94 15.67
11	OC_2H_5	H	CH	C- OCH_3	H	白色粉末	246-247	43.74 43.52	4.20 4.19	14.57 14.32
12	OC_2H_5	H	CH	N	OCH_3	棕褐色粉末	194-195	40.51 40.16	3.92 3.87	18.17 17.91
13	OC_2H_5	H	N	C- OCH_3	H	白色粉末	223-224	40.51	3.92	18.17

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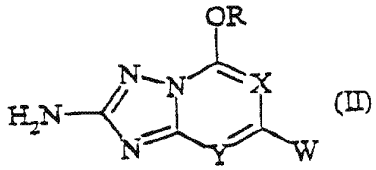
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								40.18	3.89	17.89
14	OCH_3	H	N	C- Cl	H	白色粉末	208-209	35.16 34.95	2.68 2.68	18.64 18.61
15	OCH_3	H	CH	N	OCH_3	白色粉末	218-219	38.81 38.63	3.53 3.35	18.86 18.60
16	OCH_3	H	CH	C- OCH_3	H	白色粉末	245-246	42.16 42.42	3.81 3.80	15.13 15.06
17	OCH_3	H	N	C- CH_3	H	白色粉末	230-231	40.56 40.36	3.69 3.61	19.71 19.68
18	OCH_3	Cl	N	C- OCH_3	H	棕褐色粉末	222-223	35.52 35.53	2.98 2.99	17.26 17.10
19	OCH_3	Cl	CH	N	OCH_3	白色粉末	205-206	35.52 35.16	2.98 2.90	17.26 17.23
20	OCH_3	Cl	N	C- Cl	H	白色粉末	208-210	32.21 32.14	2.21 2.21	17.07 16.97
21	OCH_3	Cl	N	C- CH_3	H	白色粉末	212-214	36.97 36.78	3.10 3.04	17.97 17.70
22	OCH_3	CH_3	N	C- Cl	H	白色粉末	220-221	36.97 37.14	3.10 3.14	17.97 17.82
23	OCH_3	CH_3	N	C- OCH_3	H	白色粉末	219-220	40.51 40.67	3.92 3.98	18.17 17.90
24	OCH_3	CH_3	CH	N	OCH_3	白色粉末	209-210	40.51 39.98	3.92 3.83	18.17 18.27
25	OCH_3	CF_3	CH	N	OCH_3	棕褐色粉末	219-221	35.54 35.66	2.75 2.78	15.94 15.31

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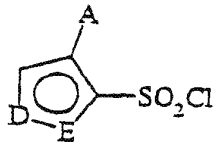
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其中 T 代表氢的式 I 化合物可通过下述方法制备:将式 II 的取代的 2-氨基[1,2,4]三唑并嘧化合物:



(II)

与式 III 的噻吩磺酰氯化合物反应



(III)

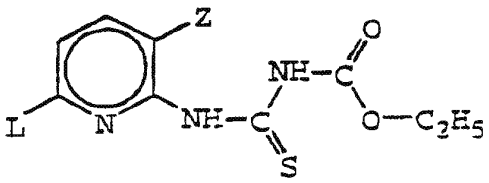
其中 A,D,E,R,W,X 和 Y 如式 I 化合物中所述定义。反应是在室温下,将近似等摩尔量的两种化合物在极性,非质子溶剂如乙腈中混合,然后加入吡啶和催化量(占磺酰氯化合物的摩尔百分数为 5-25%)二甲基亚砜的条件下进行。如需要,为完全反应,可再添加磺酰氯化合物,吡啶和二甲基亚砜。反应可进行几小时至几天以完成反应。排除水分的方法可采用如干燥氮气层的方式。获得的式 I 化合物是固体并在许多普通有机溶剂和水中溶解度低,可采用常规方式回收该化合物。

其中 T 不代表氢的式 I 的 N-(三唑并嘧基)噻吩磺酰胺化合物是将相应的其中 T 代表氢的式 I 化合物在相关的磺酰胺酰化反应技术领域中已知的反应条件下通过酰化获得的。适合的酰化剂包括烷酰氯化合物,如丙酰氯或三氟乙酰氯;氯甲酸酯化合物,如氯甲酸 2-甲氧基乙酯;氨基甲酰氯化合物,如 N',N'-二烯丙基氨基甲酰氯以及异氰酸烷基酯化合物,如异氰酸 2-氯乙酯。

在三唑并嘧环上带有烷氧基取代基的式 I 化合物可采用本领域已知的用于上述置换反应的常规方法将相应的卤代的式 I 化合物用适合的醇盐处理而制备。通常在三唑并嘧环系的某些位置上的卤素取代基比其它位置上的卤素取代基更易于替换而且可选择地替换。

许多式 II 的 2-氨基[1,2,4]三唑并[1,5-a]吡啶化合物(X 代表 C-H 和 Y 代表 C-Z)可由下式的适当取代的 N-(2-吡啶基)-N'-羰乙氧基硫脲化合物与羟胺反应获得的:

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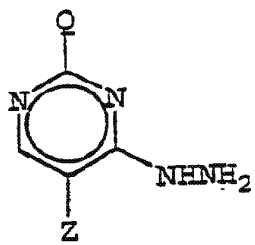


其中 Z 如式 I 化合物中所述定义以及 L 是 OR 或卤素。该反应通常是在溶剂如乙醇中进行并需要加热几个小时。羟胺通常是用受阻叔胺如二异丙基乙胺或碱金属醇盐如乙醇钠中和盐酸而产生的。式 II 目标化合物可采用常规方法回收,如通过蒸发除去反应混合物中的挥发性组分,并可通过常规方法纯化,如用水和/或其它溶剂萃取,其中上述化合物微溶于水和/或其它溶剂。作为该方法的起始物的 N-(2-吡啶基)-N'-羰乙氧基硫脲化合物可通过将适当取代的 2-氨基吡啶化合物用乙氧羰基异硫氰酸酯处理而获得。通常反应是在室温下,惰性有机溶剂中进行。详尽方法进一步公开在美国专利 5,571,775 中。

用作上述方法的起始物的取代的 2-氨基吡啶化合物是本领域已知的或可通过本发明公开的方法或本领域已知的常规方法制备。

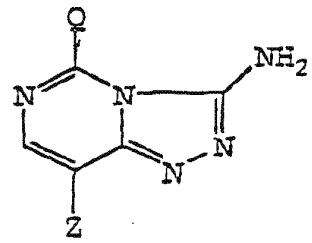
其中 X 代表 C-H 的式 II 化合物还可通过 B.Vercek 等人在 Monatshefte fur Chemie,114,789-798(1983)中公开的方法由适当取代的 2-氰基氨基吡啶化合物制备。上述化合物的其它制备方法公开在 K.T.Potts 等人的 Journal of Organic Chemistry,31,265-273(1966)中。

其中 X 代表 N 的式 II 化合物即 2-氨基[1,2,4]三唑并[1,5-c]嘧啶化合物,可由下式的 4-肼基嘧啶化合物制备:



其中 Q 代表甲硫基或氯以及 Z 如式 I 化合物中所述定义。首先将肼基嘧啶化合物用溴化氰处理获得下式的 3-氨基-8-取代-5-取代[1,2,4]三唑并[4,3-c]-嘧啶化合物的氢溴化物:

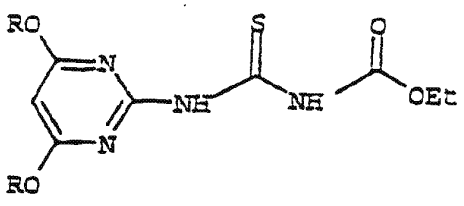
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其中 Q 代表甲硫基或氯以及 Z 如式 I 化合物中所述定义。反应通常是在室温下,有机溶剂,如异丙醇中进行。产物可通过常规方法回收,如通过加入非极性溶剂,例如二乙醚,并收集过滤后形成的固体。然后通过用碱金属醇化物如甲醇钠或乙醇钾以及丙烯酸乙酯在作为溶剂的相应的醇中处理,将其中 Q 代表甲硫基的上述中间体转化为其中 Q 代表烷氧基的式 II 目标化合物。化合物重排并且甲硫基部分被得自介质醇的烷氧基部分所替换。通常反应是在低于 25℃下进行。式 II 的目标化合物可通过乙酸中和回收并收集通过过滤或其它常规方法获得的固体。其中 X 代表 N 以及 Q 代表氯的式 II 化合物可通过将相应的其中 Q 代表氯的[4,3-c]中间体用三烷基胺碱异构化获得。用作这些方法的起始物的 4-肼基嘧啶化合物通过将本领域公知的相应的 4-氯嘧啶化合物与肼反应获得。

其中 X 代表 N 的式 II 化合物的其它制备方法公开在 G.W.Miller 等人的 J.Chemical Society,1965,第 3357 页以及 1963,第 5642 页中。

其中 Y 代表 N 的式 II 化合物,例如 2-氨基[1,2,4]三唑并[1,5-a]嘧啶化合物,可通过将下式的 N-(4,6-二烷氧基嘧啶-2-基)-N'-羰乙氧基硫脲与羟胺反应制备获得:



其中 R 如式 I 中所述定义。通常反应是在溶剂如乙醇中进行并需要加热几个小时。羟胺通常是用受阻叔胺如二异丙基乙胺或碱金属醇盐如乙醇钠中和盐酸而产生的。式 II 目标化合物可采用常规方法回收,如通过蒸发除去反应混合物中的挥发性组分,并可通过常规方法纯化,

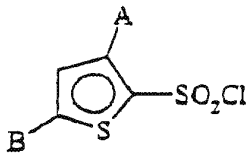
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如用水和/或其它溶剂萃取,其中上述化合物微溶于水和/或其它溶剂。作为该方法的起始物的 N-(4,6-二烷氧基嘧啶-2-基)-N'-羰乙氧基硫脲可通过将 2-氨基-4,6-二烷氧基嘧啶用乙氧羰基异硫氰酸酯处理而获得。通常反应是在室温下,惰性有机溶剂中进行。详尽方法进一步公开在美国专利 5,571,775 中。

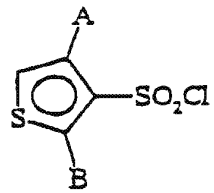
用于上述方法的 2-氨基-4,6-二烷氧基嘧啶起始物在本领域是已知的。

式 III 的取代的噻吩磺酰氯起始物可通过本发明公开的方法或本领域已知的通用或特定方法制备。许多上述化合物可通过下述方法制备:将相应的噻吩化合物用丁基锂锂化,将获得的噻吩基锂化合物与 SO_2 反应,然后用 N-氯琥珀酰亚胺氯化。上述反应步骤中的每一步,通常采用适于这些方法的已知条件。噻吩基锂化合物还可以通过卤代噻吩与正丁基锂的卤素-金属交换反应获得。许多丙基或苄硫基噻吩化合物还可采用标准方法将相应的巯基噻吩化合物烷基化而制备或将噻吩基锂化合物与适合的二硫化物反应获得。随后存在水的条件下,例如用氯气氯化获得所需的磺酰氯化合物。

式 III 化合物包括下式的取代的噻吩-2-磺酰氯化合物:



以及下式的取代的噻吩-3-磺酰氯化合物:



其中 A 和 B 各自独立地代表 H,卤素,CF_3,R,OR'或 CO_2R"。优选取代的噻吩-3-磺酰氯,特别是那些其中 A 或 B 是 OR'或 CO_2R"的化合物。

而且可以将式 I 的 N-(三唑并嘧基)噻吩磺酰胺化合物直接用作除草剂,优选将其用于含有除草有效量的该化合物与至少一种农业上可接受助剂或载体组成的混合物中。适合的助剂或载体应对有用作物没有植物毒性,特别是在有作物存在的情况下使用组合物进行选择性杂草防

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治时的施用浓度下不产生植物毒性以及不应与式 I 化合物或其它的组合物成分发生化学作用。可将上述混合物设计为直接施用于杂草或它们的生长区或将其制成浓缩物或制剂,通常在施用前用额外的载体和助剂稀释。它们可以是固体,如,例如粉剂,颗粒剂,水分散粒剂,或可湿性粉剂,或液体,如,例如可乳化的浓缩物,溶液,乳状液或悬浮剂。

用于制备本发明除草混合物的适合的农业助剂和载体对本领域普通技术人员来说都是公知的。

可以使用的液体载体包括水,甲苯,二甲苯,石脑油,作物油,丙酮,甲基乙基酮,环己酮,三氯乙烯,全氯乙烯,乙酸乙酯,乙酸戊酯,乙酸丁酯,丙二醇一甲基醚以及二甘醇一甲基醚,甲醇,乙醇,异丙醇,戊醇,乙二醇,丙二醇,甘油,N-甲基-2-吡咯烷酮等。通常选择水作为稀释浓缩物的载体。

适合的固体载体包括滑石,叶蜡石粘土(pyrophyllite clay),硅石,一种美国活性白土(attapulgus clay),硅藻土(kieselguhr),白垩,硅藻土(diatomaceous earth),石灰,碳酸钙,膨润土,漂白土,棉籽壳,小麦粉,豆粉,浮石,木粉,胡桃壳粉,木素等。

通常有利的是向本发明组合物中加入一种或多种表面活性剂。上述表面活性剂可有利地用于固体和液体组合物,特别是那些设计为需要在施用前用载体稀释的组合物中。表面活性剂的性质可以是阴离子,阳离子或非离子型以及可以用作乳化剂,湿润剂,悬浮剂,或其它目的。代表性的表面活性剂包括烷基硫酸盐,如月桂基二乙醇硫酸铵;烷芳基磺酸盐,如十二烷基苯磺酸钙;烷基苯酚-烯化氧加成产物,如壬基苯酚-C_{18} 乙氧基化物;醇-烯化氧加成产物,如十三烷醇-C_{16} 乙氧基化物;皂类,如硬脂酸钠;烷基萘磺酸盐,如二丁基萘磺酸钠;二烷基酯磺基琥珀酸盐,如二(2-乙基己基)磺基琥珀酸钠;山梨糖醇酯,如山梨糖醇油酸酯;季铵,如月桂基三甲基氯化铵;脂肪酸的聚乙二醇酯,如硬脂酸聚乙二醇酯;环氧乙烷和环氧丙烷的嵌段共聚物;以及一烷基磷酸酯盐和二烷基磷酸酯盐。

常用于农用组合物中的其它助剂包括相容性试剂,消泡剂,螯合剂,中和剂和缓冲剂,缓蚀剂,染料,香料,铺展剂,渗透剂,粘着剂,分散剂,增稠剂,防冻剂,杀微生物剂等。组合物中还可以含有其它相容组分,例如,其它除草剂,除草剂安全剂,植物生长调节剂,杀菌剂,杀虫剂等以及可与液体肥料或固体粒状肥料载体如硝酸铵,尿素等一起进行配制。

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本发明除草组合物中的活性成分浓度通常以重量计为 0.001-98 重量%。常常采用的浓度为 0.01-90 重量%。制成浓缩物形式使用的组合物中,活性成分通常是以 5-98 重量%,优选 10-90 重量%浓度存在。通常将上述组合物,在施用前用惰性载体,如水稀释。通常施用至杂草或杂草的生长区的稀释组合物中一般含有 0.001-5 重量%并优选含有 0.01-0.5%的活性成分。

通过应用常规的地面或空中喷粉机,喷雾器,以及颗粒撒施机,通过加入灌溉水中,以及本领域技术人员已知的其它常规手段将本发明组合物施用至杂草或它们的生长区。

已发现式 I 化合物可用于出苗前(包括种植前)以及出苗后除草剂。通常优选出苗后施用。该化合物可有效地防治阔叶和禾本科杂草。尽管式 I 包括的各 N-(三唑并嗪基)噻吩磺酰胺化合物都在本发明范围内,然而获得的除草活性,作物选择性,以及杂草防治谱的程度是根据取代基及存在的其它特性而变化。可以使用较高的,非选择性施用量的上述化合物用于防治基本上区域内的所有植物。某些情况下,还可以使用较低的,选择性施用量的上述化合物用以防治禾本科作物或阔叶作物中的不需要的植物。在某些情况下,经常通过使用安全剂提高选择性。

本发明使用的术语除草剂表示可防治或负面调节植物生长的活性成分。除草有效量或控制植物生长量是指可产生负调节效应以及包括偏离自然生长发育,杀灭,调节,干燥,阻滞作用等的活性成分量。术语植物意指包括发芽的种子,出苗的幼苗以及定居的各种植物。

当将本发明化合物在任何生长阶段或种植或出苗前直接施用至植物或植物生长区时均表现出除草活性。观察到的效果随下述条件而变化:需防治的植物种类,植物的生长期,稀释和喷雾液滴大小的施用参数,固体组分的粒径,使用时的环境条件,使用的具体化合物,使用的特定助剂和载体,土壤类型等,以及化学品的施用量。作为本领域技术人员公知的,可调整上述及其它因素以促进非选择性或选择性除草作用。通常,优选将式 I 化合物在出苗后施用至相对未发育成熟的植物以获得最佳的除草效果。

通常在出苗后施用时采用的施用量为 0.001-1 Kg/Ha;出苗前施用时,通常用量为 0.01-2 Kg/ha。通常较高用量时可获得非选择性防治广谱

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的不需要植物。而在较低用量下通常可获得选择性防治效果,并通过适当选择化合物,施用时间以及施用量可用于作物生长区。

本发明化合物(式 I)常与一种或多种其它除草剂联合使用以防治更广泛种类的不需要植物。当与其它除草剂联用时,可将本发明要求保护的化合物与其它除草剂配制成制剂,与其它除草剂的罐混形式,或与其它除草剂顺次使用。有利于与本发明化合物混合使用的一些除草剂包括取代的三唑并嘧啶磺酰胺化合物,如唑嘧磺胺,唑嘧磺胺盐和氯唑啶草。还可以使用其它除草剂如三氟羧草醚,噻草平,氯嘧磺隆,异噁草松,乳氟禾草灵,氟酮唑草,fumiclorac,氟草隆,氟磺胺草醚,咪唑喹啉酸,咪唑乙烟酸,利谷隆,嗪草酮,吡氟禾草灵,氟吡禾灵,草甘膦,草铵膦,2,4-D,乙草胺,异丙甲草胺,稀禾啶,烟嘧磺隆,二氯吡啶酸,氯氟吡氧乙酸,甲磺隆,酰嘧磺隆,苯磺隆,以及其它除草剂。通常优选将上述化合物与具有类似作物选择性的其它除草剂联合使用。通常还优选以混剂或罐混形式同时施用各除草剂。

通常本发明化合物可与广泛类型的已知除草剂安全剂混合使用以提高它们的选择性,如喹氧乙酸,吡咯二酸,解草噁唑,烯丙酰草胺,解草嗪,解草胺,氟草肟,香草隆,哌草丹,禾草丹,以及解草啶。具有通过提高细胞色素 P-450 氧化酶活性改进植物体内对除草剂的代谢作用的除草剂安全剂通常是特别有效的。这通常是本发明的优选实施方案。本发明化合物还可用于防治通过遗传操纵或突变及选择获得的已对除草剂产生耐药性或抗性的许多作物中的不需要植物。例如,可以处理已对普通除草剂或对抑制敏感植物中的乙酰乳酸合成酶的除草剂产生耐药性或抗性的作物。

实施例

下述实施例将对本发明各个方面进行解释。

1.制备 4-溴-3-羟基-2-噻吩羧酸甲酯

45 分钟(min)内向 31 克(0.2 摩尔)3-羟基-2-噻吩羧酸甲酯的 400 毫升(ml)冰醋酸溶液中加入 31.3 克(0.2 摩尔)Br_2。16 小时(hr)后,6 小时内加入 31.3 克 Br_2 并将反应物再搅拌 18 小时。将反应混合物倒入 $NaHSO_3$ 水溶液中并用醚萃取。分离有机相,用水(2 x 300 毫升)洗涤,$MgSO_4$ 干燥,

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过滤并浓缩成粘性油。将上述油(47 克)溶解在 200 毫升甲醇中,15℃下 48 小时后,通过过滤收集到 36 克(77%)的浅粉红色的结晶。熔点 79-80 ℃。^{1}H NMR (300MHz, $CDCl_3$): δ 9.8(br, 1H); 7.4(s, 1H); 3.9(s, 3H)。对 $C_6H_5BrO_3S$ 的元素分析计算值: C, 30.04; H, 2.23; S, 13.52。实测值: C. 30.04; H, 1.92; S, 14.01。

2.制备 4-溴-3-甲氧基-2-噻吩羧酸甲酯

向 36 克(0.15 摩尔)4-溴-3-羟基-2-噻吩羧酸甲酯的 300 毫升二甲基亚砜(DMSO)溶液中加入 32 克(0.23 摩尔)甲基碘,然后加入 32 克粉状 K_2CO_3。2 小时后,将反应混合物倒入水中并用醚萃取。用水(4 x 200 毫升)洗涤有机相,$MgSO_4$ 干燥,过滤并浓缩获得 36 克无色固体。^{1}H NMR (300MHz, $CDCl_3$): δ 7.4(s, 1H); 4.0(s, 3H); 3.9(s, 3H)。对 $C_7H_7BrO_3S$ 的元素分析计算值: C, 33.48; H, 2.81; S, 12.77。实测值: C, 33.18; H, 2.70; S. 12.49。

3.制备 4-溴-2-羰基-3-甲氧基-噻吩

将 37.5 克(0.15 摩尔)4-溴-3-甲氧基-2-噻吩羧酸甲酯和 12 克 KOH(0.18 摩尔)混合物的 250 毫升甲醇和 100 毫升水组成的溶液回流 2 小时。将反应混合物冷却并真空除去大部分甲醇。将上述溶液分配于醚和稀 HCl 水溶液中。分离有机相,$MgSO_4$ 干燥,过滤,浓缩,真空干燥获得 34 克(94%)的无色固体。熔点为 191-192℃。^{1}H NMR (300MHz. $CDCl_3$): δ 7.5(s, 1H); 4.1(s, 3H)。对 $C_6H_5BrO_3S$ 的元素分析计算值: C. 30.04; H, 2.23; S, 13.52。实测值: C, 29.89; H, 2.06; S, 13.14。

4.制备 4-溴-2-羰基-5-氯-3-甲氧基噻吩

在-10℃,通过向 22.4 克(0.22 摩尔)二异丙胺的 150 毫升无水四氢呋喃(THF)溶液中加入 88.6 毫升(0.22 摩尔)的 2.5M 正丁基锂制备获得二异丙胺锂(LDA)溶液。然后在-78℃下将其缓慢加入 15 克(63 毫摩尔)4-溴-2-羰基-3-甲氧基噻吩 200 毫升无水 THF 的无色溶液中获得深红色溶液。1 小时后,加入 60 克(0.25 摩尔)C_2Cl_6,使反应混合物缓慢升温至室温。将反应混合物用醚和稀 HCl 水溶液分配。然后用 5%NaOH 水溶液萃取有机相几次。合并水提取物,用 20%HCl 水溶液酸化并用新鲜醚萃取。用 $MgSO_4$ 干燥有机层,过滤并浓缩获得 16.1 克(94%)棕褐色固体。熔点 191-192℃。^{1}H NMR (300MHz, $CDCl_3$): δ 4.1(s, 3H)。对 $C_6H_4BrClO_3S$

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的元素分析计算值: C, 26.54; H, 1.48; S, 11.81。实测值: C, 27.02; H, 1.55; S, 11.42。

5.制备 4-溴-2-羰基-3-甲氧基-5-甲基噻吩

在-10℃,通过向 25.6 克(0.26 摩尔)二异丙胺的 150 毫升无水 THF 溶液中加入 101 毫升(0.26 摩尔)2.5M 正丁基锂制备 LDA 溶液。然后在-78℃下将其缓慢加入 15 克(63 毫摩尔)4-溴-2-羰基-3-甲氧基噻吩的 200 毫升无水 THF 的无色溶液中获得深红色溶液。1 小时后,在-35℃下将上述溶液通过插管导入 60 克(0.42 摩尔)甲基碘的 300 毫升无水 THF 溶液中。将反应混合物用醚和稀 HCl 水溶液分配。将有机相用 $MgSO_4$ 干燥,过滤并浓缩获得浅褐色固体。将上述固体通过己烷和 CH_2Cl_2 混合物重结晶获得 11 克棕褐色固体。熔点 166-167℃。^1H NMR (300MHz, $CDCl_3$): δ 3.9(s, 3H); 2.3(s, 3H)。对 $C_7H_7BrO_3S$ 的元素分析计算值: C, 33.48; H, 2.81; S, 12.77。实测值: C, 33.47; H, 2.77; S, 12.59。

6.制备 3-甲氧基-5-三氟甲基-2-噻吩羧酸甲酯

向 20 克(88 毫摩尔)的 3-羟基-5-三氟甲基-2-噻吩羧酸甲酯的 200 毫升 DMSO 溶液中加入 37 克(260 毫摩尔)的甲基碘和 25 克(181 毫摩尔)的粉状 K_2CO_3。搅拌 2 小时后,将反应混合物倒入水中并用醚萃取。分离有机相,用水洗涤几次,$MgSO_4$ 干燥,过滤并浓缩获得 20 克近乎无色固体。将上述固体进一步通过 12:1 己烷:乙酸乙酯重结晶纯化获得 10.5 克无色结晶。将母液浓缩并通过柱色谱纯化获得另外 8 克产物(87%产率)。熔点为 78-79℃。^1H NMR (300MHz, $CDCl_3$): δ 7.2(s, 1H); 4.0(s, 3H); 3.9(s, 3H)。对 $C_8H_7F_3O_3S$ 的元素分析计算值: C, 40.00; H, 2.94; S, 13.35。实测值: C, 39.87; H, 2.94; S, 13.47。

7.制备 3-甲氧基-5-三氟甲基-2-噻吩羧酸

将 2 克(14 毫摩尔)甲基-3-甲氧基-5-三氟甲基-2-噻吩羧酸甲酯,2 克(28 毫摩尔)KOH 的混合物的 15 毫升水和 50 毫升甲醇溶液回流 2 小时。将反应混合物用醚和稀 HCl 水溶液分配。分离有机相,$MgSO_4$ 干燥,过滤并浓缩获得 2.8 克(87%)的无色固体。熔点为 131-132℃。^1H NMR (300MHz, $CDCl_3$): δ 7.2(s, 1H); 4.1(s, 3H)。对 $C_7H_5F_3O_3S$ 的元素分析计算值: C, 37.17; H, 2.23; S, 14.18。实测值: C, 36.96; H, 2.19; S, 14.08。

8.制备 4-苄硫基-3-甲氧基-5-三氟甲基-2-噻吩羧酸

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在-20℃,向 3 克(13 毫摩尔)3-甲氧基-5-三氟甲基-2-噻吩羧酸的 100 毫升无水 THF 溶液中加入 5.3 毫升(13 毫摩尔)的 2.5M 正丁基锂形成浆液。向上述浆液中加入刚刚通过在 50 毫升无水 THF 中加入 2.0 克(19.7 毫摩尔)二异丙胺和 7.9 毫升 2.5M 正丁基锂制备的 LDA 溶液。在 0-5℃下经 2 小时,在将 9.8 克(40 毫摩尔)二苄基二硫化物溶液作为 THF 溶液加入浆液之前,浆液逐渐形成暗色溶液。搅拌反应物并在用醚和稀 Na_2CO_3 水溶液分配前使之升温至 15℃。分离水相,用 10%HCl 水溶液酸化并用新鲜醚萃取。分离有机相,浓缩,然后通过反相高效液相色谱(HPLC)纯化,使用 50:50 乙腈与 0.5%H_3PO_4 水溶液混合物。真空除去大部分乙腈后,通过用醚萃取从适当的馏分中回收产物。分离有机相,$MgSO_4$ 干燥,过滤并浓缩获得 2 克(43%)的棕褐色固体。熔点为 146-147℃。^{1}H NMR (300MHz, $CDCl_3$): δ 9.6(br, 1H); 7.2(m, 5H); 4.12(s, 3H); 4.09(s, 2H)。对 $C_{14}H_{11}F_3O_3S_2$ 的元素分析计算值: C, 48.27; H, 3.18; S, 18.41。实测值: C, 48.00; H, 3.14; S, 18.44。

9.制备 3-苄硫基-4-甲氧基-2-三氟甲基噻吩

将 3.6 克 4-苄硫基-3-甲氧基-5-三氟甲基-2-噻吩羧酸和 0.85 克铜粉混合物的 50 毫升喹啉溶液加热至 150℃15 分钟。将混合物冷却并用醚和 HCl 水溶液分配。分离有机相,$MgSO_4$ 干燥,过滤,浓缩并通过柱色谱纯化获得 2.2 克(71%)琥珀油。^{1}H NMR (300MHz, $CDCl_3$): δ 7.2(m, 5H); 6.4(s, 1H); 4.1(s, 2H); 3.8(s, 3H)。对 $C_{13}H_{11}F_3OS_2$ 的元素分析计算值: C, 51.30; H, 3.64; S, 21.07。实测值: C, 51.13; H, 3.54; S, 21.02。

10.制备 3-溴-4-甲氧基噻吩

在 140mm 真空度下,将 15 克 4-溴-3-甲氧基-噻吩-2-羧酸样品加热至 200-220℃,维持 40 分钟。通过蒸馏收集到近乎无色的油(11 克)。沸点 160-165℃@80-85mm。将上述油通过色谱进一步纯化获得 8.8 克无色油。^{1}H NMR (300MHz, $CDCl_3$): δ 7.2(d, 1H, J=3.5); 6.2(d, 1H, J=3.5); 3.9(s, 3H)。对 C_5H_5BrOS 的元素分析计算值: C, 31.11; H, 2.61; S, 16.61。实测值: C, 30.98; H, 2.61; S, 16.89。

通过类似方法制备下述的脱羧基噻吩化合物:

3-溴-4-乙氧基噻吩

沸点 140-145℃@35mm。^{1}H NMR (300MHz, $CDCl_3$): δ 7.2(d, 1H,

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J=3.5); 6.2(d, 1H, J=3.5); 4.1(q, 2H, J=7); 1.5(t, 3H, J=7)。对 C_6H_7BrOS 的元素分析计算值: C, 34.80; H, 3.41; S, 15.48。实测值: C, 34.56; H, 3.36; S, 15.44。

3-溴-2-氯-4-甲氧基噻吩

沸点 160-165℃@10mm。熔点 63-64℃。^{1}H NMR (300MHz, $CDCl_3$): δ 6.1(s, 1H); 3.8(s, 3H)。对 $C_5H_4BrClOS$ 的元素分析计算值: C, 26.40; H, 1.77; S, 14.09。实测值: C, 26.63; H, 1.76; S, 13.95。

3-溴-4-甲氧基-2-甲基噻吩

沸点 150-155℃@10mm。^{1}H NMR (300MHz, $CDCl_3$): δ 5.9(s, 1H); 3.7(s, 3H); 2.3(s, 3H)。对 C_6H_7BrOS 的元素分析计算值: C, 34.80; H, 3.41; S, 15.48。实测值: C, 34.66; H, 3.57; S, 15.60。

2-氯-4-甲氧基噻吩

沸点 120-125℃@15mm。^{1}H NMR (300MHz, $CDCl_3$): δ 6.65(d, 1H, J=2); 6.0(d, 1H, J=2); 3.8(s, 3H)。对 C_5H_5ClOS 的元素分析计算值: C, 40.41; H, 3.20; S, 21.57。实测值: C, 40.21; H, 3.20; S, 21.43。

11.制备 5-氯-2-氯磺酰基-3-甲氧基噻吩

在-30℃,氮气氛围中,向 3.8 克(26 毫摩尔)2-氯-4-甲氧基噻吩的 100 毫升醚溶液中缓慢加入 11.2 毫升 2.5M 正丁基锂。将上述溶液短暂加热至室温,然后冷却至-60℃。然后在-30℃,将上述溶液转移至 4 克(85 毫摩尔)SO_2 的 300 毫升醚溶液中获得稠浆液。将上述浆液稀释至便于搅拌并使之升温至室温。通过过滤收集固体并在加入 75 毫升异丙醇之前将其直接溶解在 75 毫升水中。向其中分批加入 3.8 克(29 毫摩尔)的 N-氯琥珀酰亚胺。30 分钟后将溶液用醚和稀 $NaHSO_3$ 水溶液分配。分离有机相,$MgSO_4$ 干燥,浓缩并通过 HPLC 纯化获得 3.1 克琥珀油。^{1}H NMR (300MHz, $CDCl_3$): δ 6.8(s, 1H); 4.1(s, 3H)。对 $C_5H_4Cl_2O_3S_2$ 的元素分析计算值: C, 24.30; H, 1.63; S, 25.95。实测值: C, 24.10; H, 1.59; S, 25.65。

根据类似方法制备下列 2-氯磺酰噻吩化合物:

2-氯磺酰基-3-甲氧基噻吩

熔点 71-72℃。^{1}H NMR (300MHz, $CDCl_3$): δ 7.7(d, 1H, J=5.6); 7.0(d, 1H, J=5.6); 4.1(s, 3H)。对 $C_5H_5ClO_3S_2$ 的元素分析计算值: C, 28.24; H, 2.37; S, 30.15。实测值: C, 28.41; H, 2.33; S, 30.18。

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12.制备 3-氯磺酰基-2-甲氧基噻吩

在-78℃,向 9 克(47 毫摩尔)3-溴-2-甲氧基噻吩的 150 毫升无水 THF 溶液中缓慢加入 19 毫升 2.5M 正丁基锂。30 分钟后,向溶液中喷入过量的无水 SO_2 使之沉淀获得无色固体。将上述混合物升至室温,用醚稀释并通过过滤收集固体。将上述固体溶解在 150 毫升异丙醇和水的等量混合物中。向上述溶液中分批加入 6.7 克(50 毫摩尔)N-氯琥珀酰亚胺。15 分钟后,将反应溶液用醚和稀 $NaHSO_3$ 水溶液分配。分离有机相,$MgSO_4$ 干燥,过滤,浓缩并通过柱色谱纯化获得 5.1 克琥珀油。^1H NMR (300MHz, $CDCl_3$): δ 6.2(s, 1H); 3.9(s, 3H)。对 $C_5H_5ClO_3S_2$ 的元素分析计算值: C, 28.24; H, 2.37; S, 30.15。实测值: C, 28.56; H, 2.57; S, 29.96。

根据类似方法制备下列 3-氯磺酰基噻吩化合物:

3-氯磺酰基-4-甲氧基噻吩

^1H NMR (300MHz, $CDCl_3$): δ 8.1(d, 1H, J=3.8); 6.4(d, 1H, J=3.8); 4.0(s, 3H)。对 $C_5H_5ClO_3S_2$ 的元素分析计算值: C, 28.24; H, 2.37; S, 30.15。实测值: C, 28.24; H, 2.37; S, 29.85。

3-氯磺酰基-4-乙氧基噻吩

^1H NMR (300MHz, $CDCl_3$): δ 8.1(d, 1H, J=3.5); 6.4(d, 1H, J=3.5); 4.2(q, 2H, J=6.9), 1.2(t, 3H, J=6.9)。对 $C_6H_7ClO_3S_2$ 的元素分析计算值: C, 31.79; H, 3.11; S, 28.28。实测值: C, 32.07; H, 3.19; S, 27.97。

2-氯-3-氯磺酰基-4-甲氧基噻吩

^1H NMR (300MHz, $CDCl_3$): δ 6.2(s, 1H); 3.9(s, 3H)。对 $C_5H_5Cl_2O_3S_2$ 的元素分析计算值: C, 28.24; H, 2.37; S, 30.15。实测值: C, 28.56; H, 2.57; S, 29.96。

3-氯磺酰基-4-甲氧基-2-甲基噻吩

^1H NMR (300MHz, $CDCl_3$): δ 6.0(s, 1H); 3.8(s, 3H); 2.6(s, 3H)。对 $C_6H_7ClO_3S_2$ 的元素分析计算值: C, 28.24; H, 2.37; S, 30.15。实测值: C, 28.56; H, 2.57; S, 29.96。

13.制备 3-氯磺酰基-4-甲氧基-2-三氟甲基噻吩

在 0℃,向经过搅拌的 5.3 克(17.4 毫摩尔)3-苄硫基-4-甲氧基-2-三氟甲基噻吩的 250 毫升 CH_2Cl_2 和 250 毫升稀 HCl 水溶液混合物中,缓慢通

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入 4.7 克 Cl_2。将混合物加热至室温并分离有机相,$MgSO_4$ 干燥,过滤并浓缩成琥珀油。将上述油通过 HPLC 纯化获得 3 克无色油。^1H NMR (300MHz, $CDCl_3$): δ 6.6(s, 1H); 4.0(s, 3H)。对 $C_6H_4ClF_3O_3S_2$ 的元素分析计算值: C, 25.68; H, 1.44; S, 22.85。实测值: C, 25.98; H, 1.5; S, 22.65。

14.制备 3-氨基-8-氯-5-甲硫基[1,2,4]三唑并[4,3-c]嘧啶氢溴化物

室温下,边搅拌边将 40 毫升(120 毫摩尔)的 3 摩尔溴化氰的二氯甲烷溶液与 19.0 克(100 毫摩尔)5-氯-4-肼基-2-甲硫基嘧啶和 200 毫升无水异丙醇混合。将所得混合物搅拌 18 小时,然后用 500 毫升二乙醚稀释。将形成的固体经过滤回收并干燥获得理论量的目标化合物,该化合物为黄色固体,熔点大于 250℃。^1H NMR (DMSO-d_6): δ 7.80(s, 1H); 2.67(s, 3H)。^{13}C: δ 150.96, 147.90, 143.10, 138.38, 113.16, 14.22。对 $C_6H_7N_5BrClS$ 的元素分析计算值: C, 24.3; H, 2.38; N, 23.6; S, 10.8。实测值: C, 26.1; H, 2.69; N, 24.0; S, 12.2。

根据类似方法制备下述的 3-氨基[1,2,4]三唑并[4,3-c]嘧啶化合物:

3-氨基-8-甲氧基-5-甲硫基[1,2,4]三唑并[4,3-c]嘧啶氢溴化物-棕褐色固体,熔点 180-182℃; 对 $C_7H_{10}N_5BrOS$ 的元素分析计算值: C, 28.8; H, 3.45; N, 24.0; S, 11.0。实测值: C, 29.0; H, 3.44; N, 23.9; S, 11.1。

3-氨基-8-甲基-5-甲硫基[1,2,4]三唑并[4,3-c]-嘧啶氢溴化物-黄色固体,熔点为 234-236℃; 对 $C_7H_{10}N_5BrS$ 的元素分析计算值: C, 30.6; H, 3.30; N, 25.5; S, 11.7。实测值: C, 30.7; H, 3.52; N, 25.3; S, 11.5。

15.制备 2-氨基-8-氯-5-甲氧基[1,2,4]三唑并[1,5-c]嘧啶

制备 15.0 克(51 毫摩尔)3-氨基-8-氯-5-甲硫基[1,2,4]三唑并[4,3-c]嘧啶氢溴化物,8.2 毫升(76 毫摩尔)丙烯酸乙酯,以及 150 毫升甲醇的混合物并将该混合物在冰浴中冷却。在冷却和搅拌条件下,将 17 毫升(76 毫摩尔)的 4.5 摩尔甲醇钠的甲醇溶液缓慢加入上述混合物中。加完后,将混合物加热至室温并搅拌 18 小时。然后用 2.0 毫升乙酸中和。将形成的固体通过过滤回收,用二乙醚洗涤,并干燥获得 7.7 克(理论值的 75%)的目标化合物,棕褐色粉末,熔点大于 250℃。^1H NMR (DMSO-d_6): δ 8.0(s, 1H); 6.6(brs, 2H); 4.1(s, 3H)。^{13}C: δ 166.40, 151.65, 147.73, 140.95, 108.57, 56.12。对 $C_6H_6N_5ClS$ 的元素分析计算值: C, 36.1; H, 3.03; N, 35.1。实测值: C, 36.1; H, 3.19; N, 34.8。

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根据类似方法制备下列 2-氨基[1,2,4]三唑并[1,5-c]嘧啶化合物:

2-氨基-5,8-二甲氧基[1,2,4]三唑并[1,5-c]嘧啶-棕褐色粉末,熔点为 201-203℃; 对 $C_7H_9N_5O_2$ 的元素分析计算值: C, 43.1; H, 4.65; N, 35.9。实测值: C, 43.2; H, 4.67; N, 35.6。

2-氨基-8-甲基-5-甲氧基[1,2,4]三唑并[1,5-c]嘧啶-棕褐色固体,熔点大于 250℃; 对 $C_7H_9N_5O$ 的元素分析计算值: C, 46.9; H, 5.06; N, 39.1。实测值: C, 46.7; H, 4.84; N, 39.1。

16.制备 2-氨基-5,8-二甲氧基[1,2,4]三唑并[1,5-a]吡啶

将 58.4 克(0.21 摩尔)[(3,6-二甲氧基吡啶-2-基)氨基]羰硫基(carbonothioyl)氨基甲酸乙酯,70 克(1 摩尔)羟胺盐化物和 105 毫升(0.6 摩尔)二异丙基乙胺混合物的 1 升乙醇溶液加热回流 12 小时。冷却后,真空条件下将所得溶液蒸干。向剩余物中加入水(250 毫升)和二乙醚(100 毫升)并将混合物搅拌 15 分钟。通过过滤收集产物并在室温,真空干燥获得 25.2 克的 2-氨基-5,8-二甲氧基[1,2,4]三唑并[1,5-a]吡啶。熔点为 223-234℃。^{1}H NMR (DMSO-d_6): δ 6.9(d, 1H, j=8.7); 6.2(d, 1H, j=8.4); 5.9(s, 2H); 4.0(s, 3H); 3.8(s, 3H)。

17.制备 2-氨基-5,7-二甲氧基[1,2,4]三唑并[1,5-a]嘧啶

将 2-氨基-4,6-二甲氧基嘧啶(5.0 克,36 毫摩尔)溶解在无水四氢呋喃(THF,35 毫升)中,向其中加入乙氧羰基异硫氰酸盐(6.4 毫升,54 毫摩尔)并将上述溶液在室温下搅拌。24 小时后,真空条件下除去溶剂并将剩余物与醚混合形成结晶状固体。通过真空过滤移出固体并干燥获得[(4,6-二甲氧基嘧啶-2-基)氨基]羰硫基氨基甲酸乙酯,棕褐色固体(8.9 克,87%)。熔点 196-197℃。^{1}H NMR ($CDCl_3$): δ 13.2(bs, 1H); 8.8(bs, 1H); 5.80(s, 1H); 4.32-4.25(q, 2H, J=7.2); 3.93(s, 3H); 1.30(t, 3H, J=7.2)。

将[(4,6-二甲氧基嘧啶-2-基)氨基]羰硫基氨基甲酸乙酯(0.50 克,1.7 毫摩尔)与乙醇(5 毫升)混合。向上述混合物中加入羟胺盐化物(0.12 克,1.7 毫摩尔)和二异丙基乙胺(0.30 毫升,1.7 毫摩尔)。将所得混合物在室温下搅拌。2.5 小时后,向混合物中加入另外的二异丙基乙胺(0.30 毫升,1.7 毫摩尔)。48 小时后真空除去乙醇并将剩余物在 H_2O 和 Et_2O 间分配获得粉末。将该粉末过滤并干燥获得棕褐色粉末状产物(0.27 克,82%)。对 $C_7H_9N_5O_2$ 的元素分析计算值: C, 43.08; H, 4.65; N, 35.88; O,

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16.39。实测值: C, 39.88; H, 4.22; N, 32.00; O, 16.35。^{1}H NMR (DMSO-d_6): δ 6.04(s, 1H); 5.97(br, 2H); 4.04(s, 3H)。

18.制备 3-甲氧基-N-(5,8-二甲氧基[1,2,4]三唑并[1,5-c]嘧啶-2-基)噻吩-2-磺酰胺(化合物 1)

向 0.8 克(4.5 毫摩尔)2-氨基-5,8-二甲氧基[1,2,4]三唑并[1,5-c]嘧啶的 7 毫升乙腈浆液中加入 1.3 克(6.1 毫摩尔)3-甲氧基噻吩-2-磺酰氯,0.6 毫升无水吡啶和 0.003 毫升无水 DMSO。96 小时后,将反应混合物用 CH_2Cl_2 和水分配。分离有机相,用稀 HCl 水溶液洗涤,$MgSO_4$ 干燥,过滤和浓缩。将剩余物溶解在少量 CH_2Cl_2 中,之后边搅拌边缓慢加入醚以沉淀为纯棕褐色固体。过滤收集固体并真空干燥获得 0.85 克(50%)目标产物。熔点 227-228℃。^{1}H NMR (300MHz, DMSO-d_6): δ 12.1(s, 1H); 7.8(d, 1H, J=5.6); 7.6(s, 1H); 7.0(d, 1H, J=5.6); 4.1(s, 3H); 3.9(s, 3H); 3.8(s, 3H)。对 $C_{12}H_{13}N_5O_5S_2$ 的元素分析计算值: C, 38.81; H, 3.53; N, 18.86。实测值: C, 38.22; H, 3.49; N, 18.91; S, 17.34。

根据类似方法制备表 1 和 2 中的 N-([1,2,4]三唑并[1,5-c]嘧啶-2-基)噻吩磺酰胺,N-([1,2,4]三唑并[1,5-a]嘧啶-2-基)噻吩磺酰胺,以及 N-([1,2,4]三唑并[1,5-a]吡啶-2-基)噻吩磺酰胺化合物。

19.制备除草组合物可湿性粉剂

将高岭土粘土(55.5 克),Hisil 233 二氧化硅(5.0 克),Polyfon H(木质素磺酸钠;7.0 克),Stepanol ME-Dry(十二烷基硫酸钠;7.9 克),以及化合物 1(20.4 克)加入 1 夸脱玻璃制韦林氏搀合器杯中并高速充分混合。将掺合好的混合物(一次)通过实验用 Trost 磨,该磨具有设定为 75-80psi(517-551 Kpa)的对置喷嘴(opposing jets)。由此获得具有优异润湿性和悬浮性能的可湿性粉剂。通过用水稀释该可湿性粉剂可获得用于防治杂草的适宜浓度的悬浮剂。

水悬浮浓缩物

为制备水悬浮浓缩物,将去离子水(106 克),Kelzan S(黄原胶;0.3 克),Avicel CL-611(羧甲基纤维素;0.4 克),以及 Proxel GXL(1,2-苯并异噻唑啉-3-酮;0.2 克)加入掺合机中并混合 30 分钟。然后将化合物 6(44 克),Darvan #1(萘磺酸盐;2 克),Foamaster UDB(硅酮液;0.2 克),Pluronic P-105(环氧乙烷/环氧丙烷嵌段共聚物;20 克),磷酸(0.02 克),以及丙二醇

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(16 克)加入上述的掺合机中并混合 5 分钟。将已混合好的各组分在装填有 1-1.25 毫米无铅玻璃珠(40 毫升)的 Eiger 磨中以 5000rpm 研磨 30 分钟。对 Eiger 磨外部冷却,使研磨室保持在 15℃。

油基悬浮浓缩物

向 1 夸脱玻璃制韦林氏搀合器杯中加入 Exxon's 作物油(145.4 克),Amsul DMAP 60(十二烷基苯磺酸的二甲基氨基丙烷盐;4.0 克)和 Attagel 50(一种美国活性白土;4.0 克)。将混合物高速充分混合以确保均匀性。Amsul DMAP 是难以分散的,但最终形成均匀的小油珠。加入 Agrimul 70-A(乙氧基化双亚甲基辛基酚;4.0 克)和 Emulsogen M(油醇-环氧乙烷;16.0 克)并充分混合直至混合物质地均匀。然后向混合物中掺入喹氧乙酸异庚酯(cloquintocet mexyl)(5.4 克),随后加入化合物 7(21.3 克)。采用水悬浮浓缩物制备中所述条件,将最后的磨料分散体在 Eiger 磨中研磨。

20.评估出苗后除草活性

将所需测试植物种类的种子播种在表面积为 64 平方厘米的塑料盆中的 Grace-Sierra MetroMix®306 栽培混合物中,通常该混合物的 pH 为 6.0-6.8 以及有机质含量约占 30%。当需要确保获得良好的萌发条件和健康植株时,可进行杀菌剂处理和/或其它化学或物理处理。将植物在温室中生长 7-21 天,上述温室中每天保持约 15 小时的光照时间,白天约 23-29℃以及夜晚 22-28℃。定期加入营养物质和水以及如需要在顶部安装 1000 瓦金属卤化物灯补充光照。当植物长至第一或第二真叶期时应用于测试。

根据将要测试的最高用量确定的数值称重各试验化合物,将其放入 20 毫升玻璃瓶中并溶解于 4 毫升丙酮和二甲基亚砜 97:3 v/v(体积/体积)的混合物中获得浓缩储备溶液。如果测试化合物难以溶解,可将混合物加热和/或声波振荡。将获得的浓缩储备液用含有丙酮,水,异丙醇,二甲基亚砜,Atplus 411F 作物油浓缩物和 Triton X-155 表面活性剂(亚甲基双二戊基苯氧基聚乙氧基乙醇)48.5:39:10:1.5:1.0:0.02 体积/体积的混合物的水溶液稀释获得已知浓度的喷雾溶液。用于试验的最高浓度溶液是由 13 毫升混合物稀释 2 毫升等分的储备液获得的以及较低浓度溶液是通过稀释适当的更少等分的储备液获得的。采用压缩空气压力为

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2-4psi(140-280 千帕斯卡)推动的 DeVilbiss 喷雾器将已知浓度的每种溶液中约 1.5 毫升等分均匀地喷雾至每个测试植物花盆使得均匀地覆盖每株植物。采用同样方法用上述混合物水溶液喷雾对照植物。该试验中 1ppm 的施用量相当于使用约 1 克/公顷。

将处理后的植物和对照植物放在如上所述的温室中并通过在盆底部浇水以防止冲刷掉测试化合物。2 周后,通过与未处理植物比较,肉眼观察测试植物的生长状况并分为 0-100%的等级记录,其中 0 相当于对植物没有伤害以及 100 相当于全部杀死植物。某些测试化合物,施用量,测试植物种类,以及结果列于表 3 中。

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表 3

出苗后除草活性

化合物序号	使用量 ppm	STEME	XANST	CHEAL	IPOHE	AMARE	ABUTH	VIOTR	POLCO	ALOMY	SETFA	SORBI	AVEFA
1	3.9	75	50	75	85	95	75	70	98	95	100	98	99
2	7.8	98	60	90	85	98	95	95	90	70	98	98	90
3	31	70	100	95	70	100	70	80	85	70	85	90	80
4	15.6	60	60	90	60	100	70	60	90	70	85	70	60
6	7.8	80	95	80	85	100	90	85	85	80	98	80	70
7	1.9	98	80	60	80	-	80	80	-	98	100	98	98
8	7.8	95	60	70	60	-	75	75	-	50	60	95	85
9	62	95	60	40	70	-	70	60	-	60	20	70	10
10	15.6	98	100	70	80	95	90	90	70	50	90	80	80
11	15.6	80	90	95	70	100	85	75	80	75	100	90	85
12	15.6	95	90	90	95	95	95	80	85	60	95	95	85
13	7.8	95	95	90	75	100	95	75	990	80	95	95	85
14	31.3	100	95	100	80	95	90	75	95	80	95	95	80
15	15.6	90	90	90	70	90	70	70	80	80	100	100	95
16	15.6	90	95	95	60	95	80	70	90	85	90	90	80
17	31.3	90	95	100	70	95	85	70	60	70	90	90	85

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18	15.6	90	100	90	75	100	85	100	95	70	90	100	95
19	15.6	95	95	100	80	100	80	100	95	70	100	100	100
20	31.3	85	100	75	75	95	85	90	90	30	50	85	70
21	62.5	100	90	100	80	100	100	90	100	30	50	100	75
22	31.3	80	90	90	90	100	70	80	90	60	80	95	50
23	15.6	80	90	80	40	90	90	70	75	70	90	90	70
24	7.8	80	90	85	80	100	70	70	70	80	90	100	70
25	31	60	90	90	70	90	70	70	70	80	80	95	80

STEME=繁缕(*Stellaria media*)

CHEAL=红心藜(*Chenopodium album*)

AMARE=反枝苋(*Amaranthus retroflexus*)

VIOTR=三色堇(*Viola tricolor*)

ALOMY=鼠尾看麦娘(*Alopecurus myosuroides*)

SORBI=二色蜀黍(*Sorghum bicolor*)

XANST=苍耳(*Xanthium strumarium*)

IPOHE=裂叶牵牛(*Ipomoea hederacea*)

ABUTH=苘麻(*Abutilion theophrasti*)

POLCO=荞麦蔓(*Polygonum convolvulus*)

SETFA=费氏狗尾草(*Setaria faberi*)

AVEFA=野燕麦(*Avena fatua*)

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21.评估出苗前除草活性

将所需测试植物种类的种子播种在由壤土与砂子 70:30 比例混合制备的土壤基质中,上述壤土是由约 43%淤泥(silt),19%粘土,和 38%砂子组成的且 pH 值约为 8.1 以及有机质含量约为 1.5%。土壤基质是装填在表面积为 161 平方厘米的塑料盆中。当需要确保获得良好的萌发条件和健康植株时,可进行杀菌剂处理和/或其它化学或物理处理。

根据将要测试的最高用量确定的数值称重各试验化合物,将其放入 20 毫升玻璃瓶中并溶解于 8 毫升丙酮和二甲基亚砜 97:3 v/v(体积/体积)的混合物中获得浓缩储备溶液。如果测试化合物难以溶解,可将混合物加热和/或声波振荡。将获得的储备液用水和 Tween®155 表面活性剂(乙氧基化失水山梨糖醇脂肪酸酯)的 99.9:0.1 的混合物稀释获得已知浓度的使用溶液。用于试验的最高浓度溶液是由 8.5 毫升混合物稀释 4 毫升等分的储备液获得的以及较低浓度溶液是通过稀释适当的更少等分的储备液获得的。采用配有 TeeJet TN-3 空心锥体喷嘴的 Cornwall 5.0 毫升玻璃注射器将已知浓度的每种溶液的 2.5 毫升等分均匀地喷雾至每个已播种盆的土壤中使溶液完全覆盖每盆土壤。采用同样方法用上述混合物水溶液喷雾对照盆。当使用 50 毫克测试化合物时可获得 4.48 公斤/公顷的最高施用量。

将处理后的塑料盆以及对照盆放在温室中,上述温室中每天保持约 15 小时的光照时间,白天约 23-29℃以及夜晚 22-28℃。定期加入营养物质和水以及如需要在顶部安装 1000 瓦金属卤化物灯补充光照。通过顶部灌溉方式浇水。3 周后,通过与未处理植物的萌发及生长情况比较,肉眼观察测试植物的萌发及生长状况并分为 0-100%的等级记录,其中 0 相当于对植物没有伤害以及 100 相当于全部杀死植物或没有萌发。某些测试化合物,施用量,测试植物种类,以及结果列于表 4 中。

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表 4

出苗前除草活性

化合物序号	使用量 g/Ha	XANST	CHEAL	IPOHE	AMARE	ABUTH	EPHHL	ALOMY	ECHCG	DIGSA	SETFA	SORBI	AVEFA
1	17.5	70	98	85	100	98	100	100	100	100	100	100	100
2	8.8	-	98	80	95	75	90	95	75	100	100	100	100
3	35	70	95	60	95	50	70	80	70	95	90	90	70
4	35	0	80	50	100	70	40	95	0	90	90	80	60
6	35	70	99	80	100	80	70	75	80	100	100	100	80
7	4.4	80	-	85	95	95	85	100	99	100	100	100	100
8	8.8	80	70	75	80	70	85	85	25	50	60	70	80
9	70	25	-	10	40	30	10	100	0	10	15	50	30
10	8.8	-	95	80	100	70	75	90	90	70	90	100	90
11	4.4	50	95	30	90	80	50	100	10	40	100	98	70
12	8.8	90	100	80	100	90	95	100	100	100	100	100	100
13	4.4	80	100	90	90	80	100	100	100	100	100	100	100
14	35	60	98	60	80	80	30	100	98	100	100	100	100
15	8.8	60	98	70	85	90	90	100	80	100	100	100	100
16	35	40	80	0	60	40	0	100	0	30	0	0	0
17	35	70	100	0	70	30	60	100	30	30	60	60	30

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18	4.4	90	100	80	95	90	90	100	100	98	100	100	80
19	8.8	50	90	90	75	95	90	95	95	100	100	95	100
20	17.5	60	70	70	80	80	50	90	95	85	50	90	90
21	8.8	50	70	40	60	80	60	70	30	40	70	70	70
22	35	80	98	98	100	100	80	70	100	98	100	100	70
23	4.4	70	90	80	100	90	90	98	80	100	98	100	90
24	8.8	70	100	98	90	98	80	90	40	98	100	100	70
25	17.5	50	90	60	95	80	80	95	85	85	85	90	80

XANST=苍耳(*Xanthium strumarium*)

IPOHE=裂叶牵牛(*Ipomoea hederacea*)

ABUTH=苘麻(*Abutilion theophrasti*)

ALOMY=鼠尾看麦娘(*Alopecurus myosuroides*)

DIGSA=马唐(*Digitaria sanguinalis*)

SORBI=二色蜀黍(*Sorghum bicolor*)

CHEAL=红心藜(*Chenopodium album*)

AMARE=反枝苋(*Amaranthus retroflexus*)

EPHHL=猩猩草(*Euphorbia heterophylla*)

ECHCG=稗草(*Echinochloa crus-galli*)

SETFA=费氏狗尾草(*Setaria faberi*)

AVEFA=野燕麦(*Avena fatua*)

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N-([1,2,4] triazoloazinyl) thiophene sulfonamide compounds as herbicides

Abstract

N-(Triazoloazinyl) thiophenesulfonamide compounds were prepared from appropriately substituted 2-amino[1,2,4]triazolo[1,5-c]pyrimidine, 2-amino[1,2,4]triazolo[1,5-a]pyrimidine and 2-amino[1,2, 4]triazolo[1,5-a]pyridine compounds and appropriately substituted thiophenesulfonyl chloride compounds. The compounds were found to be useful as herbicides.

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Carnitine Biosynthesis

β-HYDROXYLATION OF TRIMETHYLLYSINE BY AN α-KETOGLUTARATE-DEPENDENT MITOCHONDRIAL DIOXYGENASE*

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Rat liver mitochondria were found to hydroxylate ε-*N*-trimethyl-L-lysine to produce β-hydroxy-ε-*N*-trimethyl-L-lysine, an intermediate in carnitine biosynthesis. The hydroxylating system requires α-ketoglutarate, Fe^{2+}, and ascorbate, but does not require NADPH nor NADH. No activity was found in the microsomal or soluble fractions of liver extracts. The hydroxylated α-amino acid was isolated and characterized by column chromatography using Dowex 50-H$^+$ and Chelex 100-Cu^{2+} resins and by high voltage paper electrophoresis. The enzymatically produced β-hydroxy-ε-*N*-trimethyl-L-lysine was shown to be periodate-sensitive and one periodation product was characterized as γ-butyrobetaine aldehyde. The hydroxylated product was acted upon by crystalline serine transhydroxymethylase (EC 2.1.2.1) to yield γ-butyrobetaine aldehyde and glycine. Conversion of about 40% of the ε-*N*-trimethyl-L-lysine to β-hydroxy-ε-*N*-trimethyl-L-lysine was accomplished by this system with little or no further metabolism.

It is well documented that L-carnitine plays an important role in the translocation of both short and long chain fatty acids across the mitochondrial membrane to facilitate fatty acid synthesis and degradation (1, 2). Although carnitine is not a vitamin for most animals, the pathway by which it is synthesized has not been fully elucidated. It has been shown that the carbon chain and quaternary nitrogen atom are derived from C_{6-4} and the ε-nitrogen of lysine and that the methyl groups are derived from methionine via the *S*-adenosylmethionine system (3, 4). Thus ε-*N*-trimethyl-L-lysine is an established precursor of γ-butyrobetaine (4-*N*-trimethylaminobutyric acid) which is in turn hydroxylated to yield L-carnitine (5). Rochaix and Henderson (6) reported that the two-carbon fragment lost from the trimethyllysine is glycine and proposed the pathway for the conversion of trimethyllysine to carnitine as illustrated in Fig. 1.

Trimethyllysine has been used as an effective precursor of carnitine in whole animal experiments (7, 8) but to date, with one exception, reports have indicated no more than 2% of the dose having been converted to products in experiments using individual tissues. The exception was a reported 17% conversion of trimethyllysine to γ-butyrobetaine by kidney slices, a tissue incapable of converting γ-butyrobetaine to carnitine (9, 10). This study has been designed to locate an enzyme responsible for the hydroxylation of trimethyllysine as postulated in Fig. 1. Rat liver was chosen as the tissue for this isolation, and such hydroxylating activity has been located in the mitochondria.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats (200 to 300 g) were purchased from Bio-Lab Corp., White Bear Lake, Minn. Hepes (4-(2-hydroxyethyl)-1-piperazinemethanesulfonic acid), α-ketoglutarate, sodium ascorbate, ε-*N*-acetyl-L-lysine, bovine serum albumin, NADPH, NADH, catalase (EC 1.11.1.6), and digitonin were purchased from Sigma Chemical Co. 4-Aminobutyraldehyde diethyl acetal and iodomethane were purchased from Aldrich Chemical Co. The Dowex 50-X8 (200 to 400 mesh) and Chelex 100 (200 to 400 mesh) were purchased from Bio-Rad Laboratories and the [*methyl*-^{14}C]iodide (58 μCi/μmol) was obtained from Amersham/Searle Corp. ε-*N*-Trimethyl-L-lysine was synthesized from [*methyl*-^{14}C]iodide and α-*N*-acetyl-L-lysine using the method described by Maxwell and Lennon (11). γ-[*Methyl*-^{14}C]butyrobetaine and γ-*N*-[*methyl*-^{14}C]trimethylaminobutyraldehyde were made using the same methylation procedure starting with 4-aminobutyric acid and 4-aminobutyraldehyde diethyl acetal, respectively. Crystalline serine transhydroxymethylase was a generous gift of LaVerne Schirch, Bluffton College, Bluffton, Ohio.

Mitochondria Isolation and Incubation—Rats, fed *ad libitum* Purina rat chow, were decapitated and their livers were quickly removed and placed on ice. The liver was weighed, cut into small pieces, and forced through a Harvard Apparatus tissue press. Three volumes of the isolation buffer, containing 0.25 M sucrose, 0.05% bovine serum albumin, 2.0 mM Hepes,[1] pH 6.8, were added to the liver mince and homogenized with a motor-driven loose fitting Potter-Elvehjem homogenizer making two passes. The remainder of the procedure was basically as described by Schnaitman and Greenawalt (12): the mitochondria were pelleted at 8900 × *g*. The microsomes were obtained by further centrifugation at 100,000 × *g*. The mitochondria were washed and resuspended in isolation medium so that 3 ml contained the mitochondria from 1 g of liver; this suspension was used directly in the mitochondrial incubations.

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[1] The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazinemethanesulfonic acid.

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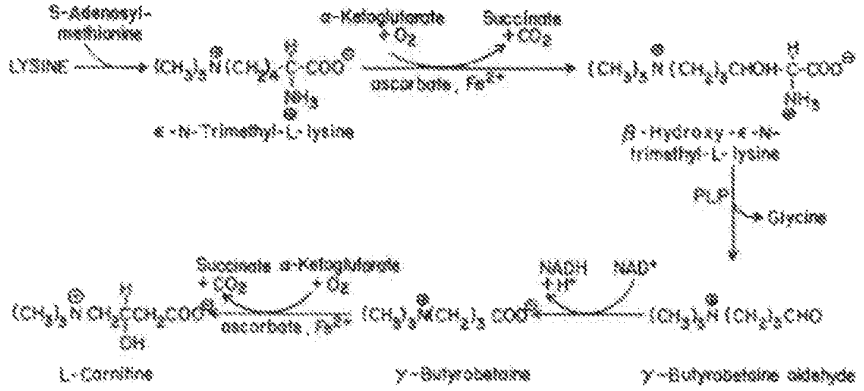


FIG. 1. Proposed biosynthetic pathway of carnitine.

The incubation mixtures contained 2.8 mM sodium ascorbate, 0.9 mM α-ketoglutarate, 0.15 mM ferrous sulfate, and 0.1 mM ε-*N*-trimethyllysine with a specific activity of 8.27 μCi/μmol in 3.0 ml of mitochondrial suspension. The α-ketoglutarate was in a 0.1 M phosphate buffer, pH 7.8, and the $FeSO_4$ and sodium ascorbate solutions were made up fresh for each incubation. The mixture was allowed to incubate for 90 min in a 37° shaking water bath at which time an equal volume of absolute ethanol was added to stop the reaction. The precipitate was removed by centrifugation and washed once with a minimal volume (~0.5 ml) of 50% ethanol. The combined supernatant and washing were fractionated on a Dowex 50-H⁺ column.

Separation of ^{14}C Products.—Glass columns (1.4 × 50.0 cm) were packed with Dowex 50-H⁺ resin and washed with H_2O. The ethanol-treated sample from the incubation mixture was placed on the column and developed with (a) 150 ml of H_2O, (b) 400 ml of 1.5 N HCl, and (c) a linear gradient using 250 ml each of 1.5 N and 3.0 N HCl. The trimethyllysine metabolites were well separated as shown in Fig. 2.

The Chelex 100 resin formed a complex with Cu^{++} and the ammonia ligand was added as described by Goldstein (15). Short (2.5 cm) columns (diameter, 1.1 cm) were packed with the resin which was then washed well with H_2O. The sample was applied and the column was developed with: (a) two 5.0-ml portions of H_2O, (b) two 5.0-ml portions of 3 N NH_4OH, and (c) two 5.0-ml portions of 0.5 N NaCl. The water wash contained compounds such as γ-butyrobetaine and carnitine which were not α-amino acids and which had insufficient net positive charge to allow them to adhere ionically to uncomplexed iminoacetic acid groups of the resin. The ammonia displaced α-amino acids such as trimethyllysine and β-hydroxy-ε-*N*-trimethyl-L-lysine (hydroxytrimethyllysine) as ligands to the copper complex. The salt wash eluted metabolites such as γ-butyrobetaine aldehyde that were ionically attached to the column and not displaced by ammonia. Samples of each eluant were counted by liquid scintillation spectrometry using scintillation fluid containing 40% absolute ethanol and 60% toluene with 4.0 g of 2,5-diphenyloxazole (PPO) and 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP)/liter.

For paper electrophoresis the samples were spotted at one end of a sheet of Whatman No. 3MM paper and electrophoresed toward the cathode using a Savant high voltage electrophoresis apparatus. The buffer used was 0.05 M acetic acid adjusted to pH 5.1 with pyridine and the sheets were electrophoresed for 30 min at 3000 V. The sheets were cut into strips and the radioactivity was located using a Packard radiochromatogram scanner which gave profiles such as that shown in Fig. 3.

For periodation of the reaction mixture 5.0 mM $NaIO_4$, 10.0 mM $NaHCO_3$, and sample in a ratio of 3:5:5 were used. The reaction was allowed to proceed for 5 min then an aliquot was quickly placed on a Chelex or Dowex 50 column or on Whatman 3MM paper and dried.

RESULTS

Initial attempts to detect the enzyme which hydroxylates trimethyllysine revealed no activity in the microsomal or soluble fractions but considerable activity was located in the mitochondrial fraction of rat liver and kidney. Further treatment of the mitochondria with 0.01% Triton X-100, a surfactant, for 1.0 h to partially destroy the integrity of the mito-

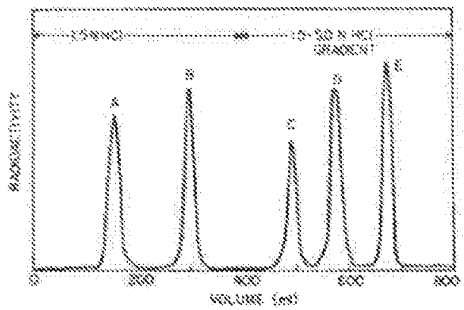


FIG. 2. Elution profile of standard trimethyllysine metabolites from a Dowex 50-H⁺ column. A sample containing γ-(*methyl*-^{14}C)carnitine (*A*), γ-(*methyl*-^{14}C)butyrobetaine (*B*), α-*N*-acetyl-ε-*N*-(*methyl*-^{14}C)trimethyl-L-lysine (*C*), the major metabolite from the mitochondrial incubation (*D*), and ε-*N*-(*methyl*-^{14}C)trimethyl-L-lysine (*E*) was added to a Dowex 50-H⁺ column (1.4 × 50.0 cm) and developed as described in the text.

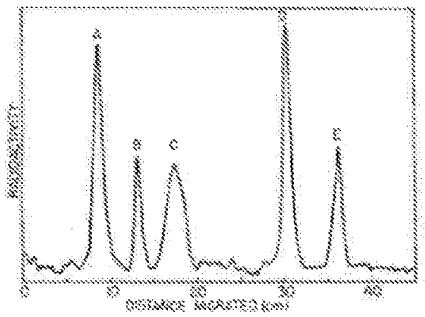


FIG. 3. Strip scan of standard trimethyllysine metabolites after high voltage paper electrophoresis at pH 5.1. Standard α-*N*-acetyl-ε-*N*-(*methyl*-^{14}C)trimethyl-L-lysine (*A*), L-(*methyl*-^{14}C)carnitine (*B*), γ-(*methyl*-^{14}C)butyrobetaine (*C*), ε-*N*-(*methyl*-^{14}C)trimethyl-L-lysine and its enzymatic hydroxylation product (*D*), and γ-(*methyl*-^{14}C)butyrobetaine aldehyde (*E*) were spotted on Whatman No. 3MM paper and electrophoresed at 3000 V for 90 min at pH 5.1. Radioactivity was detected using a Packard radiochromatogram scanner.

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chondrial membrane resulted in a loss of about two-thirds of the activity. Many different variations and combinations of cofactors were used in an attempt to optimize the conversion. The most complete conversion, approximately 40%, was obtained using the components as listed above, and referred to here as the "standard" incubation mixture. Table I shows the conversion obtained with the standard mixture and with several variations used to illustrate the cofactor requirements. Table I also contains data indicating essentially no conversion by the supernatant fraction from the mitochondrial preparation which contained the soluble and microsomal enzymes of the liver. The deletion of α-ketoglutarate, ferrous iron, or ascorbate resulted in no conversion. If the α-ketoglutarate were acting merely because of its ability to penetrate the mitochondria, and there become a source of reducing power, citrate would be expected to be suitable as well, but only a minor conversion was detected when α-ketoglutarate was replaced by citrate. Lysing the mitochondria by suspending them in 2.0 mM Hepes, pH 6.8, without sucrose did not reduce their activity. The failure of NADH or NADPH to replace α-ketoglutarate in the lysed mitochondrial system emphasizes the specificity of α-ketoglutarate as a direct electron donor for the hydroxylation reaction. It was also observed that dithiothreitol could not replace ascorbate in the reaction while other experiments, not shown here, indicated that higher concentrations of ascorbate were inhibitory. The results of preliminary experiments using digitonin to release selectively the outer membranes of the mitochondria, indicated that the hydroxylase is located in the outer membrane. Acetone powder preparations of the mitochondria were active when suspended in 2.0 mM Hepes buffer, pH 6.8.

A typical elution profile of the Dowex 50 fractionation of an incubation mixture is shown in Fig. 4. A minor peak was observed at the elution volume corresponding to standard γ-butyrobetaine (*A*) (compare Fig. 2). The major metabolite (*B*) eluted from the column during the 1.5 to 5.0 N HCl gradient at a volume between that of standard trimethyllysine (*C*) and ε-*N*-acetyl-ε-*N*-trimethyllysine (485 ml). It has been reported (16) that β-hydroxy-ε-*N*-trimethyl-α-lysine elutes from a similar column at a volume just after ε-*N*-acetyltrimethyllysine, a major metabolite from whole animal experiments. An hydroxylated product is also consistent with the postulated scheme (Fig. 1) and with the hydroxylation cofactors required in the incubation mixture. To characterize this component the appropriate tubes were pooled and the HCl was removed by lyophilization. The elution of this metabolite from a Chelex 100-Cu^{++} column by 8 N NH_4OH (Table II) indicated that it was an α-amino acid. During paper electrophoresis the metabolite co-migrated with trimethyllysine which might be expected because of the similarity in structure of trimethyllysine and hydroxytrimethyllysine. Attempts to separate the two compounds by high voltage paper electrophoresis at both acidic and basic pH values have not been successful.

Periodate treatment of the mitochondrial product resulted in one or two new products, depending on the concentration of periodate and the reaction time. Following the procedure described above, a product was obtained that remained on the Chelex 100-Cu^{++} column during both the H_2O and NH_4OH washes, but was eluted from the column with 0.5 N NaCl (Table II). This suggested a periodation product that was not an α-amino acid, as was the metabolite prior to periodation, but was a product with sufficient net positive charge to adhere to the column. The expected periodation product of hydroxytrimethyllysine, γ-butyrylaldehyde, would be expected to exhibit this behavior on the Chelex column. Also, it is important to note (Table II) that it was not simple oxidation of the α-amino acid during periodate treatment that resulted in this change since the similar α-amino acid, trimethyllysine, was unaffected by periodation.

This periodation product was characterized by paper electro-

Table I

Products from incubation of trimethyllysine with mitochondria

The standard consists of a mixture identical with that described under "Experimental Procedures." The supernatant fraction has 3.0 ml of the supernatant from the first 8300 rpm centrifugation (soluble proteins and microsomes) in place of the 3.0 ml of mitochondrial suspension. In the three mixtures where one component has been omitted 2 mM Hepes, pH 6.8, has been added to keep the volume constant. The citrate used is the same concentration as the α-ketoglutarate replaced. The Hepes samples contain mitochondria resuspended in 2 mM Hepes, pH 6.8 (not containing bovine serum albumin or sucrose). The NADH and NADPH solutions used to replace α-ketoglutarate were 0.01 M and the dithiothreitol solution used to replace ascorbate was 0.33 M.

Variation in incubation mixture	Per cent of metabolite recovered after 90 min incubation at*		
	Trimethyllysine	Hydroxytrimethyllysine	γ-Butyrobetaine and carnitine†
Standard	60.9	34.1	4.9
Supernatant fraction	99.3	0.71	
Omit α-ketoglutarate	100		
Omit $FeSO_4$	99.2	0.69	
Omit sodium ascorbate	100		
Citrate replaced α-ketoglutarate	99.2	0.79	
Hepes (lysed mitochondria)	55.9	39.2	4.85
Hepes, NADH replaced α-ketoglutarate	100		
Hepes, NADPH replaced α-ketoglutarate	100		
Dithiothreitol replaced ascorbate	99.8	0.2	

* These are per cents of total radioactivity recovered; the total corresponds to 93 to 99% of the dose.

† Both carnitine and γ-butyrobetaine elute from the column in the 1.5 N HCl wash and were not separated in these experiments.

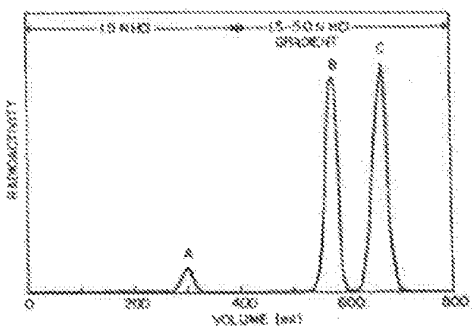


FIG. 4. Typical Dowex 50-H⁺ elution profile of mitochondrial incubation mixture. The supernatant from the ethanol-treated reaction mixture was added to a Dowex 50-H⁺ column (1.4 × 50.0 cm) and developed as described in the text. *Peak A* eluted at a volume corresponding with standard γ-butyrobetaine. *Peak B* has been identified as β-hydroxy-ε-*N*-trimethyl-α-lysine, and *Peak C* elutes at a volume corresponding with standard ε-*N*-trimethyl-α-lysine, the natural substrate.

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TABLE II

Separation of trimethyllysine metabolites on a Chelex 100-Cu^{++} column

Approximately 30,000 dpm each of carnitine and mitochondrial product (*β*-hydroxy-ε-*N*-trimethyl-L-lysine) and 53,000 dpm each of γ-butyrobetaine and ε-*N*-trimethyl-L-lysine were placed on separate Chelex 100-Cu^{++} columns and developed with 6 ml each of (a) H_2O, (b) 3 N NH_4OH, (c) 0.5 N NaCl. Duplicates of the four samples were periodated using 10 μl of sample, 10 μl of 0.01 M $NaIO_4$, and 4 μl of 5.0 mM $NaIO_4$, allowing 5 min for the reaction to occur before placing them on columns and developing as above.

Sample	Peak dpm in		
	H_2O	NH_4OH	NaCl
Carnitine	54,800	400	810
γ-Butyrobetaine	52,250	200	325
ε-*N*-Trimethyl-L-lysine	190	52,600	190
Mitochondrial product	350	35,300	120
Carnitine + IO_4^-	32,700	525	1,000
γ-Butyrobetaine + IO_4^-	52,870	170	60
ε-*N*-Trimethyl-L-lysine + IO_4^-	300	52,400	455
Mitochondrial product + IO_4^-	1,670	2,000	32,150

phoresis and the results are shown in Fig. 5. The mitochondrial product that comigrated with trimethyllysine (*B*, Fig. 5*B*) prior to periodation, migrated slightly faster after periodation (Fig. 5*A*). Hydroxytrimethyllysine and γ-butyrobetaine aldehyde both have a net charge of approximately +1 at pH 3.1 but are sufficiently dissimilar that they might be expected to separate under these conditions.

The periodation product was also fractionated on a Dowex 50-H^+ column, but it appeared at the same peak effluent volume in the 1.5 N HCl eluate as did γ-butyrobetaine. Pooling the fractions in this peak and subjecting them to paper electrophoresis give a separation of the aldehyde (38 cm) and γ-butyrobetaine (19 cm). When higher concentrations of periodate (0.5 M) were used the product obtained was different. It behaved like γ-butyrobetaine on the Chelex 100-Cu^{++} column, paper electrophoresis and Dowex 50-H^+ column. γ-Butyrobetaine is the product Hoppel *et al.* (14) reported finding upon periodation of hydroxytrimethyllysine.

To identify further the periodation product, γ-*N*-[methyl-^{14}C]trimethyl aminobutyraldehyde (γ-butyrobetaine aldehyde) was synthesized and used as a standard. It would not elute from the Chelex 100-Cu^{++} column with either H_2O or 3 N NH_4OH but was eluted with 0.5 N NaCl. From a Dowex 50-H^+ column, it appeared in the same peak effluent volume as γ-butyrobetaine and it migrated about 38 cm during electrophoresis at pH 3.1. Thus, it behaved in an identical manner to the periodated mitochondrial product in all three systems.

The mitochondrial hydroxylation product, tentatively identified as hydroxytrimethyllysine, is a substrate for crystalline serine transhydroxymethylase. The product of this reaction has been identified as γ-butyrobetaine aldehyde using the Chelex 100-Cu^{++} and Dowex 50 columns, and high voltage paper electrophoresis. Thus, three distinct methods have been used to produce γ-butyrobetaine aldehyde. This aldehyde is oxidized by $KMnO_4$ to yield a product identified by high voltage paper electrophoresis as γ-butyrobetaine (Fig. 5*C*).

To substantiate further the scheme shown in Fig. 1, ε-*N*-trimethyl-L-[*carboxy*-^{14}C]lysine (6) was used to produce [*carboxy*-^{14}C]hydroxytrimethyllysine. This hydroxytrimethyllysine was incubated with crystalline serine transhydroxymethylase for 90 min and then nonradioactive glycine was added

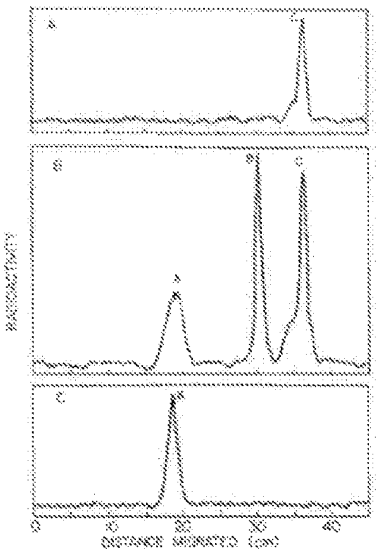


FIG. 5. Electrophoresis patterns of the [methyl-^{14}C]hydroxytrimethyllysine following treatment with periodate (*A*) and following oxidation of the periodate product with permanganate (*C*). *B* depicts the migration of authentic γ-butyrobetaine. *Peak A*, unreacted hydroxytrimethyllysine. *Peak B*, and periodate treated hydroxytrimethyllysine. *Peak C*.

TABLE III

Metabolism of carnitine intermediates by a rat liver homogenate

One gram of rat liver was homogenized with 2.0 ml of 2 mM Hepes buffer, pH 6.8, and 80 μl of 0.1 M sodium ascorbate. 100 μl of 0.1 M α-ketoglutarate, and 100 μl of 5.0 mM ferrous sulfate were added. Either 0.25 μmol of ε-*N*-trimethyllysine or 0.25 μmol of *β*-hydroxy-ε-*N*-trimethyllysine (both had specific activity of 0.27 μCi/μmol) was added, the sample was incubated at 37° for 3 h, and then the reaction was stopped with ethanol and products were resolved on a Dowex 50-H^+ column as previously described.

Substrate	% recovered as			
	ε-*N*-Trimethyllysine	Hydroxytrimethyllysine	γ-Butyrobetaine	Carnitine
ε-*N*-Trimethyllysine	100	0	0	0
β-Hydroxy-ε-*N*-trimethyllysine	0	39.7	57.8	2.5

to the reaction mixture. Glycine was isolated and recrystallized from the mixture, using aqueous ethanol, to constant specific activity, thus confirming that glycine was the two carbon fragment released by serine transhydroxymethylase.

To demonstrate the physiological significance of hydroxytrimethyllysine as a carnitine precursor it was incubated with a crude rat liver homogenate and the results are shown in Table III. Hydroxytrimethyllysine was readily converted to γ-butyrobetaine and a minimal amount of carnitine. In contrast this crude system did not act on trimethyllysine although all of the hydroxylating cofactors had been added.

DISCUSSION

ε-*N*-Trimethyl-L-lysine is an established precursor of L-carnitine, but it has been difficult to obtain an isolated organ or otherwise simplified system that would take up and metabolize trimethyllysine as does the whole animal. It appears that

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impermeability of the cell to trimethyllysine is the reason for this apparent metabolic inertness. The preliminary experiments for this report showed that lengthy exposure of either liver or kidney slices to trimethyllysine resulted in very little uptake of the substrate. This is consistent with the reports of others (9, 10). Perfusions of liver and kidney for 2-h periods during which the tissue was continually exposed to the substrate by recirculation through the vascular system also resulted in little or no conversion. On the other hand, LaBadie *et al.* (15) have done liver perfusion experiments using ^{14}C-methylated asialofetuin, which is readily taken up by the liver and hydrolyzed to yield intracellular trimethyllysine. They found 30% of the added isotope in carnitine after 90 min of perfusion. In the experiments reported here, destruction of liver or kidney cells and subsequent isolation of mitochondria also resulted in a system that effected a significant conversion of trimethyllysine to β-hydroxy-ε-*N*-trimethyl-L-lysine, when appropriate hydroxylating cofactors were added.

This hydroxylation does not make use of the most common cofactors of hydroxylating systems, NADH or NADPH, nor is it associated with the microsomal fraction as is more common for mixed function oxidases. Similar α-ketoglutarate-dependent systems have been reported for the hydroxylation in animals of γ-butyrobetaine yielding carnitine (16), of lysyl and prolyl residues in collagen precursors (17, 18), and in *Neurospora crassa* of pyrimidine deoxyribonucleosides (19), and of three sequential reactions on thymine yielding uracil-5-carboxylic acid (20). This appears to be the first α-ketoglutarate-dependent dioxygenase found in the mitochondria. Studies of Lindstedt and Lindstedt (16) on the γ-butyrobetaine hydroxylase demonstrated increased activity when catalase was added to the system; this is most likely due to destruction of H_2O_2 produced by Fe^{2+}-catalyzed autooxidation of ascorbate (21). Although no stimulation from adding catalase was observed in this study, the decrease in activity noted when the concentration of ascorbate was increased to the levels used by Lindstedt and Lindstedt (16), could be the result of enzyme inactivation by H_2O_2.

The possibility that α-ketoglutarate functions merely because it is capable of entering mitochondria where it is subsequently converted to succinate, producing NADH for hydroxylations, was considered. As has been shown in Table I, another tricarboxylic acid cycle intermediate, citrate, could not replace α-ketoglutarate. Although citrate would be converted to α-ketoglutarate via the tricarboxylic acid cycle, the α-ketoglutarate so produced would not accumulate to high enough concentrations to promote hydroxylation. However, the NADH so produced would be at approximately the same concentration as if arising from α-ketoglutarate oxidation. Mitochondrial fragments which functioned with added α-ketoglutarate were inactive when NADH or NADPH replaced α-ketoglutarate. α-Ketoglutarate therefore seems to be an essential component of this reaction, functioning as described in the previously mentioned α-ketoglutarate-dependent hydroxylases. Also consistent with this type of hydroxylation are the requirements of Fe^{2+} and ascorbate. In characterizing the α-ketoglutarate dioxygenases listed above, the α-ketoglutarate and Fe^{2+} requirements have been found to be highly specific (22-26). Reducing agents other than ascorbate can function, but ascorbate is more effective in each of the reactions.

The product of the mitochondrial incubations has been identified as β-hydroxy-ε-*N*-trimethyllysine using Dowex 50-H^+ columns, Chelex 100-Cu^{2+} columns and periodation followed by paper electrophoresis and Chelex 100-Cu^{2+} columns. Hoppel *et al.* (14) isolated a small amount of hydroxytrimethyllysine from large volumes of urine from rats injected with large doses of trimethyllysine. They reported its elution from a Dowex 50-H^+ column between trimethyllysine and α-*N*-acetyltrimethyllysine; this is the same relative position as the product described here, although a somewhat different elution procedure was used. It has been shown that the product of this hydroxylation is an α-amino acid by its retention on a Chelex 100-Cu^{2+} column and subsequent elution with 3 N NH_4OH. The periodate sensitivity of this compound has been demonstrated using mild enough conditions that α-amino acids would not be affected. These data indicate that the hydroxylating system used has produced a metabolite which has a hydroxyl group in a vicinal position to the α-amino group. Further characterization by paper electrophoresis and Chelex 100-Cu^{2+} chromatography of the periodation product has led to the conclusion that it is γ-butyrobetaine aldehyde and hence the product of mitochondrial enzyme is hydroxytrimethyllysine. Although periodation of hydroxytrimethyllysine has been reported to yield γ-butyrobetaine (14), milder conditions have been shown here to give the aldehyde. The identity of the periodation product has been verified by synthesis of the aldehyde then comparing its behavior during Chelex 100-Cu^{2+} and Dowex 50-H^+ column chromatography and on high voltage paper electrophoresis. The physiological significance has been demonstrated by showing production of the same aldehyde by reaction of hydroxytrimethyllysine with crystalline serine transhydroxymethylase.

This report is the first to present an isolated system from liver that efficiently converts trimethyllysine to another precursor of carnitine. Cox and Hoppel (10) reported a kidney slice system that yielded 16% γ-butyrobetaine from trimethyllysine, but this tissue did not catalyze the next reaction to produce carnitine (9, 10, 27). Hydroxylation of trimethyllysine by other tissues, including liver, which is also capable of hydroxylating γ-butyrobetaine, has been tried, but only 1 to 2% or less of the trimethyllysine has been metabolized (9, 10). The conversion reported here is near 40% using hydroxylating cofactors. The hydroxytrimethyllysine thus produced has also been shown to be an effective precursor for carnitine as demonstrated by the 60% conversion to carnitine and its immediate precursors in a 3-h experiment with crude rat liver homogenates. Indicative of the problem previously encountered, the same crude homogenate system was totally ineffective in metabolizing trimethyllysine to any other carnitine precursor, possibly reflecting the sensitivity of the enzyme to the proteases. One report (28) suggests another scheme by which trimethyllysine might be converted to carnitine, but this work was done with *Neurospora crassa* and the data reported here are not consistent with that scheme.

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Characterization of α-Ketoglutarate-dependent Taurine Dioxygenase from *Escherichia coli**

(Received for publication, May 23, 1997, and in revised form, June 23, 1997)

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The *Escherichia coli tauD* gene is required for the utilization of taurine (2-aminoethanesulfonic acid) as a sulfur source and is expressed only under conditions of sulfate starvation. The sequence relatedness of the TauD protein to the α-ketoglutarate-dependent 2,4-dichlorophenoxyacetate dioxygenase of *Alcaligenes eutrophus* suggested that TauD is an α-ketoglutarate-dependent dioxygenase catalyzing the oxygenolytic release of sulfite from taurine (van der Ploeg, J. R., Weiss, M. A., Saller, E., Nashimoto, H., Saito, N., Kertesz, M. A., and Leisinger, T. (1996) *J. Bacteriol.* 178, 5438–5446). TauD was overexpressed in *E. coli* to ~70% of the total soluble protein and purified to apparent homogeneity by a simple two-step procedure. The apparent M_r of 81,000 of the native protein and the subunit M_r of 37,400 were consistent with a homodimeric structure. The pure enzyme converted taurine to sulfite and aminoacetaldehyde, which was identified by high pressure liquid chromatography after enzymatic conversion to ethanolamine. The reaction also consumed equimolar amounts of oxygen and α-ketoglutarate; ferrous iron was absolutely required for activity; and ascorbate stimulated the reaction. The properties and amino acid sequence of this enzyme thus define it as a new member of the α-ketoglutarate-dependent dioxygenase family. The pure enzyme showed maximal activity at pH 6.9 and retained activity on storage at −20 °C for several weeks. Taurine (K_m = 55 μM) was the preferred substrate, but pentanesulfonic acid, 3-(*N*-morpholino)propanesulfonic acid, and 1,3-dioxo-2-isoindolinoethanesulfonic acid were also desulfonated at significant rates. Among the cosubstrates tested, only α-ketoglutarate (K_m = 11 μM) supported significant dioxygenase activity.

In the absence of sulfate, *Escherichia coli* can utilize aliphatic sulfonates as sulfur sources for growth. Sulfonates known to provide sulfur include ethanesulfonate, butanesulfonate, L-cysteate, isethionate (2-hydroxyethanesulfonate), and taurine (2-aminoethanesulfonate) (1, 2). None of these sulfonates served as sulfur source under anaerobic conditions, nor could they be utilized as a source of carbon and energy or of carbon, energy, and sulfur under either aerobic or anaerobic conditions (1). The mechanisms of sulfur assimilation from aliphatic sulfonates are unknown, but it has been shown that sulfonate-sulfur enters the assimilatory sulfate reduction pathway at the stage of sulfite (2).

Recently, we have identified the *tauABCD* gene cluster, located at 3.5 min on the *E. coli* chromosome, which is specifically involved in the utilization of taurine as a sulfur source (2). Disruption of *tauB*, *tauC*, or *tauD* resulted in the loss of the ability to utilize taurine as a source of sulfur, but did not affect the utilization of a range of other aliphatic sulfonates as sulfur sources. The *tau* genes were only expressed during growth in the absence of sulfate or cysteine (2). The amino acid sequences of TauABC exhibit similarity to components of ABC-type transport systems (4). TauA has a putative signal sequence, indicating that it functions as a periplasmic binding protein, and the sequences of TauB and TauC are significantly similar to those of ATP-binding proteins and membrane components, respectively, of members of the ABC transporter superfamily. It thus appears that the proteins encoded by *tauABC* constitute an uptake system for taurine. The *tauD* gene product shows 30% sequence identity to a characterized α-ketoglutarate-dependent 2,4-dichlorophenoxyacetate dioxygenase of *Alcaligenes eutrophus*, encoded by the *tfdA* gene of plasmid pJP4 (5, 6). This suggests that TauD is an α-KG[1]-dependent dioxygenase involved in the oxygenolytic release of sulfite from taurine. Here we report the purification of the TauD protein and describe its catalytic properties, which demonstrate that it is an α-KG-dependent taurine dioxygenase.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and T4 DNA ligase were obtained from MBI Fermentas. DNase I and NADH came from Boehringer Mannheim, *Pfu* DNA polymerase from Stratagene, and horse liver alcohol dehydrogenase from Sigma. Lysozyme was from Fluka, as were taurine and all other sulfonated substrates except *N*-phenyltaurine, 4-phenyl-1-butanesulfonic acid, and 3-bromoethanesulfonic acid (Sigma) and isethionic acid (Aldrich). All other chemicals were from Aldrich, Fluka, or Merck and were of the highest purity grade available.

Bacterial Strains and Growth Conditions—*E. coli* strains DH5α (7) and BL21(DE3) (8) were grown aerobically at 37 or 30 °C in Luria-Bertani medium (7) with constant shaking (180 rpm). When necessary, kanamycin was added at 50 μg/ml and ampicillin at 100 μg/ml. Solid media were prepared by the addition of 1.5% (w/v) agar.

Taurine Desulfonation in Extracts of E. coli Cells Grown in Sulfate and Taurine—*E. coli* strain MC4100 (9) was grown under the conditions mentioned above in 100 ml of a modified sulfur-free M63 minimal medium (2) containing 250 μM Na_2SO_4 or taurine as sulfur sources. When the cultures had reached an A_{600} of 0.8, cells were harvested by centrifugation at 5000 × *g* for 30 min at 4 °C, washed once with modified M63 minimal medium, resuspended in 1 ml of 20 mM Tris-HCl buffer (pH 7.0), and stored at −20 °C until further use. Cell-free extracts were obtained after the addition of 5 μg/ml lysozyme and 10 μg/ml DNase I to the cells, incubation at 30 °C for 30 min, and clarification by centrifugation at 35,000 × *g* for 20 min.

Construction of a tauD Expression Plasmid—The *tauD* gene was placed under the control of the T7 RNA polymerase promoter of vector pET-24d(+) (Novagen) in a three-step cloning procedure. First, an NdeI site at the translation start of *tauD* was introduced by polymerase chain reaction amplification of the *tauD* gene from plasmid pUC18AL.A4 (2),

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[1] The abbreviations used are: α-KG, α-ketoglutarate; HPLC, high pressure liquid chromatography; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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10). The oligonucleotide primers used were ER1 (5′-CATTGGAGAAGTCATATGATTGAAC-3′, with the change to introduce the *Nde*I site underlined) and ER2 (5′-CCGTTGCTTGAAAGCTTAGGTTCGA-3′). In the second step, the 1041-base pair polymerase chain reaction product was digested with *Nde*I and *Sph*I, and the resulting 220-base pair fragment encoding the N-terminal part of TauD was cloned in pUC18 (11), generating plasmid pME4140. In the third step, the final cloning of *tauD* in the expression vector pET 24a(+) was done by a ligation involving the vector, the 226-base pair *Nde*I-*Sph*I fragment of pME4140, and an 813-base pair *Sph*I-*Hin*dIII fragment encoding the C-terminal part of TauD. This resulted in plasmid pME4141, in which *tauD* is under the control of the T7 RNA polymerase promoter.

Purification of TauD—For protein production, *E. coli* BL21(DE3) cells harboring the taurine dioxygenase expression plasmid pME4141 were grown at 30 °C in a 5-liter Erlenmeyer flask containing 1000 ml of growth medium. When the culture had reached an A_{600} of 0.8–0.9, *tauD* expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a 1 mM final concentration and incubated for a further 3 h. Cells were collected by centrifugation at 8000 × *g* for 20 min at 4 °C, resuspended in 50 ml of 20 mM Tris-HCl buffer (pH 7.6), and stored at −20 °C in 10-ml aliquots until further use. About 6–9 g of cells (fresh weight) were collected from 1000 ml of culture.

For cell lysis and DNA digestion, 5 μg/ml lysozyme and 10 μg/ml DNase I were added to 10 ml of cell suspension, and the mixture was incubated at 30 °C with constant shaking (180 rpm) for 30 min. Crude cell-free extracts were obtained by centrifugation of the lysate at 35,000 × *g* for 20 min at 4 °C. The extract was then fractionated with solid ammonium sulfate at 4 °C. The TauD protein precipitated between 30 and 60% (w/v) $(NH_4)_2SO_4$ and was collected by centrifugation at 35,000 × *g* for 30 min at 4 °C. The precipitate was dissolved in 3.5 ml of 20 mM sodium phosphate buffer (pH 7.0) and dialyzed for 15 h at 4 °C against 1 liter of the same buffer, with gentle stirring.

The dissolved ammonium sulfate precipitate was chromatographed at room temperature on a 1-ml Resource Q anion-exchange column (Pharmacia Biotech Inc.) with a BioCAD SPRINT™ apparatus (PerSeptive Biosystems Inc.) at a flow rate of 2 ml/min. Proteins were eluted using a three-step gradient of NaCl in 20 mM sodium phosphate buffer (pH 8.6): 2 column volumes with NaCl increasing from 0 to 60 mM, 5 column volumes with 60 mM NaCl, and 2 column volumes with NaCl increasing from 60 to 1000 mM. Fractions containing active TauD were pooled, concentrated with a Centriprep 10 concentrator (Amicon, Inc.), adjusted to 10% (v/v) glycerol, and stored at −20 °C until further use.

Enzyme Assay—Taurine dioxygenase activity was assayed at 30 °C by using Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)), which produces a bright yellow color upon reaction with sulfite (12, 13). The assay mixture (1-ml final volume) contained 300 μM taurine, 1 mM α-KG, 100 μM $FeII(SO_4)$ (freshly made), 200 μM sodium ascorbate, and appropriate amounts of TauD in 10 mM imidazole buffer (pH 6.8). The pH of all potential substrate and cosubstrate stock solutions was adjusted to 6.8. Reactions were started by the addition of enzyme and stopped by the addition of 800-μl samples to a spectrophotometric cuvette containing 100 μl of EDTA (0.5 M), 100 μl of 5,5′-dithiobis(2-nitrobenzoic acid) (3 mg/ml in 100 mM sodium phosphate buffer (pH 7.0)), and 100 μl of deionized water. The colorimetric reaction was allowed to develop at room temperature for 5 min. To correct for unspecific reactions with enzyme thiol groups, formaldehyde was added (5 mM final concentration) to samples containing both sulfite and thiols. This led to complexation of the sulfite as formaldehyde sulfonate (15), after which it did not react with 5,5′-dithiobis(2-nitrobenzoic acid). Residual absorbance at 412 nm in these samples was due to thiols only and was used to make the appropriate correction. Sulfite calibration curves were prepared daily from fresh solutions of sodium sulfite in deionized water. Sulfite determination with Ellman's reagent was linear up to 110 μM sulfite. One unit of activity is defined as the amount of enzyme forming 1 μmol of sulfite/min at 30 °C under standard assay conditions.

The K_m values for taurine and α-KG were determined by keeping the concentration of one substrate constant at 600 μM while varying the concentration of the other substrate (25–600 μM taurine or 10–120 μM α-ketoglutarate). Sulfite production was followed over 3 min (steady-state conditions) by removal of aliquots every 15 or 30 s. Desulfination rates were calculated by regression analysis.

SDS Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed on a Mini-PROTEAN II system (Bio-Rad) with 10% polyacrylamide gels under denaturing conditions (7).

Analytical Methods—Oxygen consumption during TauD catalysis was measured with an oxygen electrode (Rank Brothers, Bottisham, Cambridgeshire, United Kingdom) under standard assay conditions in a 600-μl reaction volume. Protein concentrations were measured using the method of Bradford (14) with Bio-Rad reagent dye concentrate, following the manufacturer's instructions. Bovine serum albumin was used as a standard. The native M_r of TauD was estimated by gel filtration on Superose 12 HR 10/30 and Superose 6 HR 10/30 columns (Pharmacia Biotech Inc.), calibrated with known M_r protein standards: thyroglobulin (670,000), ferritin (440,000), catalase (232,000), aldolase (158,000), bovine serum albumin (67,000), ovalbumin (45,000), and RNase (13,700). The column was equilibrated (2 column volumes) and eluted with 50 mM sodium phosphate buffer (pH 7.2) containing 150 mM NaCl at a flow rate of 0.5 ml/min.

Analysis of Enzyme Reaction Products—Succinate was measured by an enzyme assay using a succinic acid test kit from Boehringer Mannheim, following the instructions of the manufacturer. The formation of aminoacetaldehyde, the unstable product of taurine desulfination, was indirectly and qualitatively detected by its reaction with horse liver alcohol dehydrogenase (EC 1.1.1.1) in a coupled assay, which yielded substoichiometric amounts of ethanolamine. The coupled assay (1-ml final volume) contained 1 mM taurine, 1 mM α-KG, 100 μM $FeII(SO_4)$ (freshly prepared), 200 μM sodium ascorbate, 175 mM NADH, 0.8 units of taurine dioxygenase, and 3.2 units of horse liver alcohol dehydrogenase in 50 mM sodium phosphate buffer (pH 6.8). Ethanolamine production from taurine was followed as NADH oxidation by measuring the absorbance at 340 nm with time. An assay mixture with no alcohol dehydrogenase served as a reference. The presence of ethanolamine in the samples was detected, after phenyl isothiocyanate derivatization, by HPLC co-chromatography with an authentic ethanolamine derivative. To this end, the material in 600–1800 μl samples was lyophilized and redissolved in 30 μl of 10 mM sodium phosphate buffer (pH 6.8). The dissolved material was then treated with phenyl isothiocyanate by the addition of 1 volume of absolute ethanol and 2.5 volumes of derivatization mixture (7:1:1 ethanol/triethylamine/phenyl isothiocyanate). After a 10-min reaction at room temperature, the derivatized sample was lyophilized, resuspended in 300 μl of 10 mM sodium phosphate buffer (pH 6.8), filtered (0.2 μm), and chromatographed on a C_{18}-Nucleosil column (250 × 4.6 mm, particle size of 7 μm) at a flow rate of 1 ml/min (Pharmacia Biotech HPLC system). A two-component buffer system (buffer A: 10 mM sodium phosphate buffer (pH 6.8); buffer B: 80% methanol and 20% 50 mM sodium phosphate buffer (pH 6.8)) was used with the following gradient: column equilibration for 10 min with 10% buffer B, from 0 to 100% buffer B in 10 min, and 100% buffer B for 5 min. Elution of taurine and ethanolamine derivatives was monitored by measuring the absorbance at 254 nm.

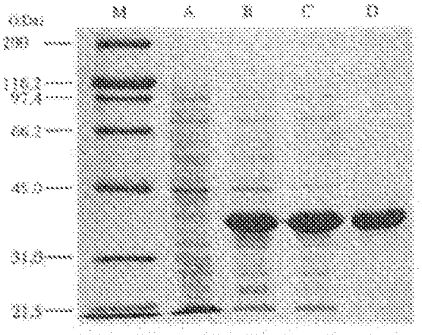


FIG. 1. **Purification of the TauD protein from *E. coli* BL21(DE3)(pME4141).** Protein samples were analyzed at different stages of the TauD purification on 10% SDS-polyacrylamide gels under reducing and denaturing conditions and stained with Coomassie Brilliant Blue. *Lane M*, molecular mass markers (indicated in kDa); *lane A*, uninduced cell extract (50 μg); *lane B*, extract of induced cells (30 μg); *lane C*, fraction precipitating between 30 and 60% ammonium sulfate (30 μg); *lane D*, purified TauD obtained after Resource Q chromatography (25 μg).

RESULTS

Desulfination of Taurine in Crude Cell Extracts—Preliminary analysis of taurine desulfination was performed with crude extract prepared from isopropyl-β-D-thiogalactopyranoside-treated cells of *E. coli* BL21(DE3)(pME4141), which strongly overexpress the *tauD* gene product (Fig. 1). Desulfination was assayed by determining the amount of sulfite formed

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Table 1
Purification of α-KG-dependent taurine dioxygenase

Purification step	Total protein	Total activity		Specific activity	Purification
		units	%		
	mg			units/mg	-fold
Cell extract	597	375	100	0.74	1
50–60% $(NH_4)_2SO_4$	196	322	62	1.22	1.6
Resource Q	65	73.8	19.7	1.84	2.2

during 15 min of incubation at 30 °C in 10 mM imidazole (pH 6.75) containing 10 mM taurine, 10 mM α-KG, 50 μM $Fe(II)SO_4$, and 100 μM L-ascorbate. Sulfite release and oxygen consumption were not detected in cell extracts prepared from uninduced cells or in extracts from induced cells incubated without taurine or α-KG. Activity in cell extracts was abolished by the addition of EDTA, and desulfonation of taurine thus required α-KG, ferrous iron, and oxygen. It therefore seemed likely that the TauD protein represents a new member of the α-KG-dependent dioxygenase enzyme family (15).

The cell extract from *E. coli* MC4100 was examined for taurine desulfonation by following taurine-dependent sulfite release. Strain MC4100 carries a single chromosomal copy of the *tau* genes, which are fully expressed during growth in sulfate-free minimal medium containing 250 μM taurine as a sulfur source (2). Extracts made from cells grown under these conditions showed a specific taurine desulfonating activity of 12.3 nmol/min/mg of protein, which corresponds to about twice the *in vivo* desulfonation rate required for growth at a doubling time of 60 min with taurine as a sulfur source. As expected, extracts from sulfate-grown cells showed no taurine desulfonating activity.

Enzyme Purification—The *tauD* gene product was purified to homogeneity from *E. coli* BL21(DE3)(pME4141) with a yield of 20% in a two-step procedure summarized in Table I. The specific taurine desulfonating activity obtained with crude extracts after overexpression was 50 times that measured in wild-type *E. coli* MC4100 cells grown in minimal medium containing taurine as a sulfur source. TauD eluted as a single symmetrical peak from the Resource Q anion-exchange column at a NaCl concentration of 15 mM. Densitometric scanning of SDS-polyacrylamide gels (Fig. 1) showed that TauD amounted to ~70% of the total soluble protein in crude extracts. Gel filtration chromatography on Superose 6 and Superose 12 HR columns was used to estimate a M_r of 81,000 ± 4400 for the native enzyme. The calculated subunit molecular mass from the *tauD* gene sequence of 32.43 kDa was estimated by SDS-polyacrylamide gel electrophoresis analysis as 37.4 ± 1.4 kDa. Since mostly mono- and dimeric (but no trimeric) structures are reported among α-KG-dependent dioxygenases (16) and with regard to the sequence identity to 2,4-dichlorophenoxyacetate dioxygenase (6), our data suggested a homodimeric structure for the TauD enzyme.

Characterization of TauD—The purified enzyme had a specific activity of 1.84 units/mg of protein. Upon storage at −20 °C at a protein concentration of 2.6 mg/ml in buffer without glycerol, >50% of the activity was lost within 3 weeks. When glycerol was added to a 15% (v/v) final concentration, the specific activity of the pure enzyme increased by ~4-fold during storage at −20 °C for 10 weeks. We presume that the activation during storage reflects folding of unfolded polypeptide chains.

The effect of pH on enzyme activity was examined over a range of 4.5–10.8 using appropriate buffer systems (17). TauD exhibited a distinct activity optimum around pH 6.9.

In our initial studies with TauD, described above, we observed that the specific activity of the enzyme fell rapidly during incubation at 30 °C. A similar effect has been seen before with the related α-KG-dependent 2,4-dichlorophenoxyacetate dioxygenase (6), so we investigated it further by measuring sulfite release from taurine over 30 min by enzyme that had been preincubated in imidazole buffer at 30 °C for 15 min alone, with $Fe(II)SO_4$, or with $Fe(II)SO_4$ and ascorbate. The data (not shown) indicate that temperature itself leads to rapid enzyme inactivation, which is enhanced by ascorbate, and that the observed inactivation is not due to oxidation of the enzyme-bound ferrous iron.

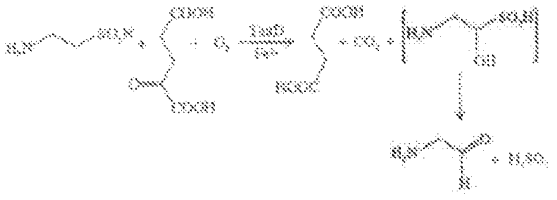


FIG. 2. Overall reaction catalyzed by the α-KG-dependent taurine dioxygenase from *E. coli*.

The dependence of TauD activity on the concentration of ferrous iron and ascorbate was investigated with pure enzyme under standard assay conditions. Enzyme activity was dependent on Fe(II) supplied as either a sulfate or chloride salt, and treatment of the enzyme with EDTA completely abolished activity. Maximal specific activity was obtained with Fe(II) concentrations between 5 and 150 μM. Other divalent metal ions, including Mg(II), Ca(II), Mn(II), Ni(II), Co(II), Cu(II), and Zn(II), could not replace ferrous sulfate at a 100 μM final concentration either as sulfate or chloride salts. Zn(II), Cu(II), and, to a lesser extent, Co(II), all added at 10–50 μM final concentrations, inhibited maximal taurine/α-KG dioxygenase activity by 80–95%. Ascorbate led to a 50% increase in activity when added at concentrations between 200 and 800 μM in an assay mixture containing 100 μM Fe(II).

Stoichiometry of the α-KG-dependent Taurine Desulfonation Reaction—Of the four products formed in the taurine/α-KG dioxygenase reaction (Fig. 2), succinate and sulfite were determined quantitatively and shown to be produced in equimolar amounts. This is in accordance with the expected stoichiometry of the reaction and demonstrated that oxidative decarboxylation of α-KG and desulfonation of taurine are strictly coupled. The expected consumption of 1 mol of dioxygen/mol of taurine desulfonated was also experimentally verified.

The putative organic product of the TauD-catalyzed taurine oxygenation is 1-hydroxy-2-aminoethanesulfonic acid, which would decompose to aminoacetaldehyde and sulfite (Fig. 2). Attempts by gas chromatography-mass spectrometry to detect aminoacetaldehyde among the products resulting from the enzymatic desulfonation of taurine were unsuccessful. Since aminoacetaldehyde is highly reactive and readily undergoes polymerization reactions, we chose an indirect method to demonstrate qualitatively its formation from taurine. As described under "Experimental Procedures," liver alcohol dehydrogenase and NADH were added to the complete reaction mixture, and reduction of the desulfonation product by alcohol dehydrogenase was monitored by following NADH oxidation. NADH oxidation occurred in the complete incubation mixture, but was not observed when taurine dioxygenase, alcohol dehydrogenase, taurine, or α-KG was omitted (Fig. 3). The identity of ethanolamine as the product indirectly formed from taurine desulfonation was verified after phenyl isothiocyanate derivatization by HPLC co-chromatography with authentic ethanolamine derivative. The dehydrogenase reaction was found to be the rate-limiting step in the coupled overall reaction. Irrespective of the concentration of alcohol dehydrogenase, only substoichiometric amounts of ethanolamine were formed from tau-

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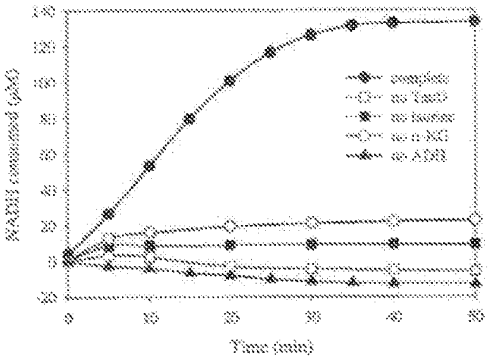
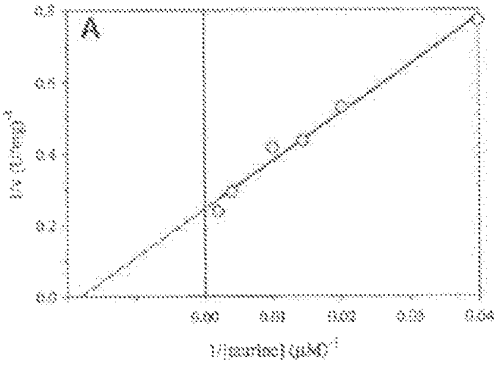


FIG. 3. **Enzymatic reduction of the product of taurine desulfonation to ethanolamine.** The formation of aminoacetaldehyde from taurine was followed by coupling the TauD-catalyzed reaction to horse liver alcohol dehydrogenase. The complete assay mixture contained (in a 1-ml volume) 1 mM taurine, 1 mM α-KG, 100 μM $Fe(II)SO_4$, 600 mM ascorbate, 175 μM NADH, 3.2 units of alcohol dehydrogenase, and 846 milliunits of TauD. NADH consumption was monitored by measuring the absorbance at 340 nm. In the controls, NADH consumption was measured in reaction mixtures lacking TauD, alcohol dehydrogenase (*ADH*), taurine, or α-KG.



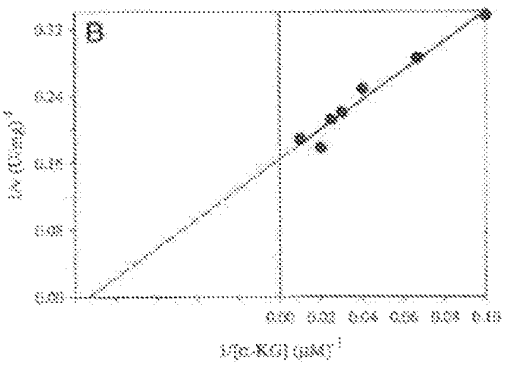


FIG. 4. **Kinetic constants of α-KG-dependent taurine dioxygenase.** The effect of taurine and α-KG concentrations on initial rates was studied by keeping the concentration of one substrate at 600 μM while varying the concentration of the other substrate (25–600 μM taurine or 10–100 μM α-KG) in the standard assay mixture. Sulfite production was followed for 3 min by withdrawing samples every 15 or 30 s, and initial rates were calculated by regression analysis. *A*, effect of taurine concentration on the rate of desulfonation; *B*, effect of α-KG concentration on the rate of desulfonation.

rine, i.e. maximally 40% of the aminoacetaldehyde produced was reduced to ethanolamine.

Substrate Range and Kinetic Constants—The enzyme showed a Michaelis-Menten-type saturation curve in response to increasing taurine concentrations, with a K_m of 55 μM and a V_{max} of 4.1 units/mg of protein (Fig. 4*A*). Among 19 potential substrates tested, methanesulfonic acid, ethanesulfonic acid, isethionic acid, 2-bromoethanesulfonic acid, L-cysteic acid, sulfosuccinate, 4-aminobenzenesulfonic acid, 2-(4-pyridyl)ethanesulfonic acid, and *N*-phenyltaurine were not utilized by TauD. 2–5% of the activity observed with taurine was observed with 1-propanesulfonic acid, 1-dodecanesulfonic acid, 4-phenyl-1-butanesulfonic acid, HEPES, and PIPES. Significant activity was supported by the compounds listed in Table II. The observed substrate range indicates that in addition to taurine, several 2-substituted ethanesulfonic acids are substrates for α-KG-dependent taurine dioxygenase. Among the unsubstituted 1-alkanesulfonates, pentanesulfonic acid was the best substrate.

The K_m value for α-KG was 11 μM (Fig. 4*B*). Using 30-min incubation times and 33 milliunits of enzyme, α-ketoadipate, pyruvate, α-ketobutyrate, α-ketovalerate, α-ketocaproate, α-ketoisovalerate, and oxaloacetate were tested as cosubstrates for taurine desulfonation. When tested at 500 μM to 10 mM, α-ketoadipate supported desulfonation at 4–10% of the rate observed with α-KG, whereas the other α-keto acids were inactive. A second carboxyl group is thus required for recognition of the cosubstrate by the enzyme.

TABLE II

Substrate range and kinetic parameters of α-KG-dependent taurine dioxygenase

Determination of K_m values was performed in 10 mM imidazole (pH 6.8), containing 600 μM α-KG, 100 μM $Fe(II)SO_4$, 600 μM sodium ascorbate, and the sulfonated substrates over the concentration range indicated.

Sulfonated substrate	K_m	V_{max}
	μM	units/mg
Taurine (25–600 μM)	55	4.1
MOPS (25–2000 μM)	143	2.0
1,3-Dioxo-2-aza-indolinoethanesulfonic acid (50–2000 μM)	360	3.5
Butanesulfonic acid (500–4000 μM)	1480	1.3
Pentanesulfonic acid (79–3000 μM)	590	1.9
Hexanesulfonic acid (300–3000 μM)	1810	2.2

DISCUSSION

Although several reports on the degradation of primary aliphatic sulfonates by bacteria as carbon or sulfur sources have appeared (1, 2, 18, 19), the enzymology of the desulfonation of these compounds is largely unexplored. The taurine desulfonation enzyme characterized in this study is, to our knowledge, the first pure enzyme reported that is capable of oxygenolytic cleavage of the C–S bond of 1-alkanesulfonates. In contrast to the partially purified bacterial monooxygenase systems for alkanesulfonate desulfonation described so far (18, 19), it does not play a role in carbon metabolism, and its synthesis is regulated by the sulfur supply to the cell (2).

The biochemical properties of taurine dioxygenase allow it to be assigned to the α-KG-dependent dioxygenase group of enzymes (15, 16). As a family, these enzymes catalyze a variety of reactions including hydroxylations, desaturations, and ring expansions. The family contains enzymes that are monomers, dimers, and tetramers with subunit sizes ranging between 26 and 86 kDa. Characterization of the reactions catalyzed by some of these enzymes revealed that not all show typical hydroxylation biochemistry in which α-KG acts as an oxygen acceptor and one atom of molecular oxygen is found in succinate and the other in the second organic product. The classifi-

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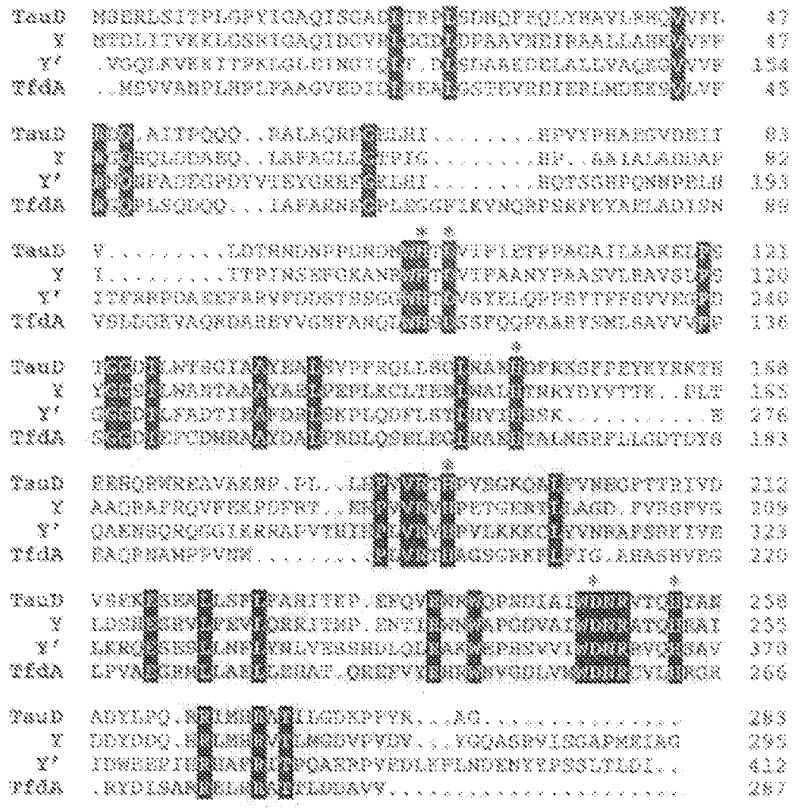


FIG. 5. **Alignment of amino acid sequences from TauD, TfdA, and related proteins.** TauD is taurine dioxygenase from *E. coli* (Swissprot accession number P37910) (3). TfdA is 2,4-dichlorophenoxyacetate dioxygenase from *A. eutrophus* (Swissprot accession number P10646) (6). Y is a hypothetical protein of unknown function from *M. tuberculosis* (GenBank™ accession number Z77185; open reading frame MTCY78.22c), and Y′ is a hypothetical protein of unknown function from *S. cerevisiae* (GenBank™ accession number Z47973, reverse complement translated from bases 12413 to 11666; only the sequence portion overlapping with the other three sequences is shown). Amino acid sequences were aligned using PILEUP (30). Conserved residues are boxed. Conserved His and Asp residues are marked with asterisks.

cation defined by biochemistry does not satisfactorily correlate with sequence data since the group contains both enzymes with similar biochemical characteristics but very little sequence similarity and enzymes with a high degree of amino acid sequence similarity but more diverse biochemical features. All these enzymes could have a common mechanism of action (16).

α-KG-dependent taurine dioxygenase exhibits a relatively relaxed substrate specificity, accepting not only taurine but also pentanesulfonic acid, hexanesulfonic acid, MOPS, and 1,3-diaza-2-isoindolineethanesulfonic acid (Table II). The first three of these alternative substrates have been shown to serve as sulfur sources for *E. coli*. Since *E. coli* mutants defective in taurine dioxygenase are still able to utilize these sulfur sources, there must be at least one additional system for their metabolism as sulfur sources (2). Desulfonation by α-KG-dependent taurine dioxygenase of organosulfonates other than taurine might thus be of little or no importance for sulfur metabolism. To strengthen or refute this view, it will be interesting to explore the specificity of the TauA periplasmic binding protein, which is coexpressed with TauD, and to characterize the system or systems that catalyze desulfonation of other alkanesulfonates.

With respect to consubstrate specificity, the taurine desulfonation enzyme of *E. coli* occupies an intermediate position among the α-KG-dependent dioxygenases. Besides α-KG, only α-ketoadipate, but none of the other α-keto acids tested, supported a low level of desulfonation. The range of consubstrates is thus more restricted than that of 2,4-dichlorophenoxyacetate dioxygenase (6), similar to the range of prolyl 4-hydroxylase (26), and less restricted than that of γ-butyrobetaine hydroxylase, which utilizes only α-KG as a cosubstrate (21).

Other features of the *E. coli* α-KG-dependent taurine dioxygenase, such as the requirement for α-KG and ferrous iron, the stimulation of activity by ascorbate, and its inhibition by divalent metal ions, are typical for α-KG-dependent dioxygenases (15, 16). Unlike lysyl hydroxylase and prolyl 4-hydroxylase, taurine dioxygenase does not appear to catalyze an uncoupled decarboxylation of α-KG in the absence of taurine. Uncoupled α-KG decarboxylation also occurs in lysyl hydroxylase and prolyl 4-hydroxylase as a side reaction during substrate hydroxylation. It is thought to lead to the oxidation of enzyme-bound Fe(II), which subsequently must be reduced by ascorbate to reactivate the enzyme. The side reaction thus results in the absolute requirement of these enzymes for ascorbate (22, 23), a property that was not observed with α-KG-dependent taurine dioxygenase.

Taurine dioxygenase exhibited 30% amino acid identity to the 2,4-dichlorophenoxyacetate dioxygenase enzyme encoded by the *tfdA* gene of plasmid pJP4 (6, 24) as well as 32 and 37% sequence identity to open reading frames of unknown function of *Saccharomyces cerevisiae* (GenBank™ accession number Z47973) and *Mycobacterium tuberculosis* (GenBank™ accession number Z77185), respectively (Fig. 5). It did not show significant similarity to other α-KG-dependent dioxygenases (16), emphasizing the genetic diversity of this group of enzymes and suggesting that they may have arisen by convergent evolution rather than from a common ancestor. While taurine dioxygenase and 2,4-dichlorophenoxyacetate dioxygenase did not react with each other's substrates,[8,9] they are both dimers, with subunit molecular masses of 32 kDa, that exhibit maximal activity at pH 6.6–7.0. The alignment of the four proteins that

[8] B. P. Hasselgrove, personal communication.
[9] E. Eichhorn, unpublished data.

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do exhibit sequence homology (Fig. 6) revealed 4 histidine and 2 aspartate residues that are strictly conserved (marked with *asterisks*). Site-directed mutagenesis studies of human prolyl 4-hydroxylase (33) and lysyl hydroxylase (26) have indicated that 2 histidines and 1 aspartate constitute the endogenous ligands of the ferrous active site. In these α-KG-dependent dioxygenases and in the mechanistically related isopenicillin-N synthase, these residues are arranged in the His-*X*-Asp-$X_{\text{n-3}}$-His motif (27), whose role in metal binding is supported by the crystal structure of the manganese form of a fungal isopenicillin-N synthase (28). Although the enzymes shown in Fig. 6 are not closely related to the above enzymes at a sequence level (<9% identity), a similar iron-binding motif might be provided by histidine 99, aspartate 101, and histidine 155 of the TauD sequence (Fig. 5). For the 2,4-dichlorophenoxyacetate dioxygenase enzyme, recent spectroscopic studies have indeed provided evidence that these residues may play a role in metal coordination (20). The function of the unknown open reading frames Y and Y′ (Fig. 5) cannot be speculated from these data, but it would be interesting to clarify if they also belong to the α-KG-dependent dioxygenase family.

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Development of 2,4-D-resistant transgenics in Indian oilseed mustard (*Brassica juncea*)

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Transgenic lines resistant to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) were developed in mustard (*Brassica juncea*), a major oilseed crop grown in more than six million hectares of land in North India. The developed construct contained the *tfdA* gene, encoding the enzyme 2,4-D monooxygenase, cloned downstream to the 35S promoter along with a leader sequence from RNA4 of alfalfa mosaic virus (AMV leader sequence), for improved expression of the transgene in plant cells. Southern analysis of T0 transgenics confirmed six out of 24 transgenics to be single copy events, from both the flanks of T-DNA. Selfed progeny derived from single copy *tfdA* lines germinated normally and rooted in medium containing 2,4-D at concentrations as high as 2.5 mg l^{-1} compared to the wild-type seedlings which did not root even at a concentration of 0.5 mg l^{-1}. The *tfdA* transgenic lines were also sprayed with commercially available 2,4-D herbicide at concentrations ranging from 10 to 1000 mg l^{-1} under field conditions. Wild type plants were affected by levels as low as 10 mg l^{-1} and were completely killed at a concentration of 50 mg l^{-1}. The four transgenic lines tested in the study were resistant to herbicide concentration of 500 mg l^{-1}. The available transgenic lines can be used for testing the potential of 2,4-D in weed control including the control of parasitic weeds (*Orobanche* spp) of mustard and for low-till cultivation of mustard.

TRANSGENIC approach has been used for improvement of *B. juncea* in the area of hybrid seed production[1,2], herbicide resistance[3] and oil quality[4]. The *bar* gene conferring resistance to herbicide phosphinothricin has been successfully deployed in *B. juncea* either alone[5] or in constructs containing *barnase* and *barstar* genes[1,2]. In both cases, *bar* gene was used as a selectable marker and enabled both *in vitro* and field-level selection of transgenic plants. However, phosphinothricin and its commercial product Basta, are expensive herbicides and their availability is rather limited in India. It will therefore be useful to test the use of 2,4-dichlorophenoxyacetic acid (2,4-D) as a herbicide for selection of transgenic plants.

2,4-D and several related phenoxy compounds have been used extensively for more than 50 years to control broadleaf weeds. It is a post-emergence, translocatable herbicide and specific only to broadleaf plants. Soil organisms that degrade 2,4-D were identified more than forty years ago[5] and the multi-enzyme pathways for 2,4-D degradation have subsequently been demonstrated in several bacterial genera[6–8]. The first gene (*tfdA*) involved in the 2,4-D degradation pathway of soil organism *Alcaligenes eutrophus*, encodes 2,4-D monooxygenase enzyme, which converts 2,4-D into less toxic 2,4-dichlorophenol and glyoxylate by cleavage of the aliphatic side chain[9]. Transgenic plants resistant to 2,4-D have been developed in tobacco as a model system[10,11] and in cotton[12,13].

In this communication, we report the development of 2,4-D-resistant transgenics in Indian mustard (*B. juncea* cv. Varuna), an agronomically important broadleaf crop, by introduction of *tfdA* gene. The single copy transgenic lines obtained were grown in a containment net-house and tested for their resistance to 2,4-D herbicide. Limited small scale trials showed that the transgenic lines were resistant to ten times higher level of herbicide tested compared to the wild-type cultivar Varuna tested under field conditions. The *tfdA* gene could provide an additional herbicide resistance field marker for use along with gene to be introgressed in transgenic crop, e.g. in the *barnase–barstar* system.

A binary vector containing the *tfdA* gene (864 bp; obtained from plasmid pRRL7) under the transcriptional control of CaMV35S promoter was developed (Figure 1 *a*). The start codon of *tfdA* gene was changed from GTG to ATG by PCR-based site-directed mutagenesis. Further, an AMV leader[14] and consensus Kozak[15] sequence were incorporated for enhanced expression of the transgene in plants. The 35S–AMV–*tfdA*–35SpA expression cassette thus constructed was finally cloned in binary vector[16] pPZP200 (ref. 16), which also contained a nosPr–*npdI*–ocspA [neomycin phosphotransferase gene (*npdI*) driven by nos promoter and carrying ocs poly A signal at 3′ end] cassette as an additional marker gene cloned within the lox sites (Figure 1 *a*). The above binary vector was transformed into disarmed *Agrobacterium tumefaciens* strain GV3101 by electroporation[17] and used for genetic transformation.

Genetic transformation of hypocotyl explants derived from *Brassica juncea* cv. Varuna was carried out following the protocols described earlier[1,3]. In two transformation experiments, around 700 explants were infected with disarmed *Agrobacterium* strain GV3101 containing the binary plasmid with *tfdA* gene. Shoots regenerated from the hypocotyl explants at an overall transformation frequency of 8.5 and 11.2% in two different experiments (Table 1). Transgenics were rooted in kanamycin-containing medium and maintained *in vitro* as nodal cultures. Forty independent transgenic plants were transferred to soil during October–November in a containment net-house as per the guidelines of the Department of Biotechnology, New Delhi.

Southern analysis of putative transgenics was carried out by isolating total genomic DNA from leaves of T0-

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grown plants using the CTAB method[18]. A 2.3 kb nosPr–*npt*II–ocspA fragment and a 200 bp *tfdA* fragment were used as probes for analysis of copy number on left and right border flanks of T-DNA respectively (Figure 1 *a*). Of the 24 transgenics subjected to analysis by Southern hybridization, six were found to be single copy integration events at both the T-DNA flanks. A representative Southern blot is shown in Figure 1 *b*. All the T0 transgenics with single copy insertion were selfed and backcrossed for their maintenance.

The T1 seeds of a representative *tfdA* line (*tfdA* 1.32) were germinated on medium containing varying concentrations (0, 0.5, 1.0, 2.5 and 5 mg l^{-1}) of 2,4-D (Sigma) along with non-transformed control Varuna (Figure 2). Both transformed and non-transformed lines showed normal germination and expansion of cotyledons up to four days. However after the fourth day, seedlings of non-transformed plants and null-segregants showed poor hypocotyl elongation on 0.5 mg l^{-1} of 2,4-D concentration. At higher concentrations of 2,4-D (> 1.0 mg l^{-1}) no hypocotyl elongation was observed even up to 10 days. Elongation of roots was inhibited even at the lowest 2,4-D concentration that was tested (0.5 mg l^{-1}). The roots remain stunted and had high density of root hair.

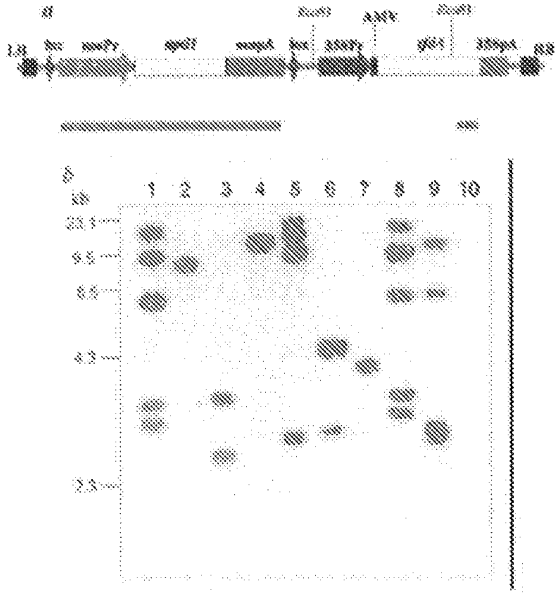


Figure 1. *a*, Schematic representation of T-DNA region of the *tfdA* construct showing the location of *Eco*RI site used for genomic DNA digestion along with the probe fragments used for the Southern analysis (hatched boxes). *b*, Southern blot of T0 transgenics showing the T-DNA integration pattern for left T-DNA border when probed with nosPr–*npt*II–pA cassette. DNA (10 µg) was digested with *Eco*RI and electrophoresed on a 0.8% agarose gel. DNA fragments were transferred onto nylon membrane (Hybond N+) and hybridized with the probe DNA labelled with ^{32}P-dCTP using the Megaprime DNA labelling kit (Amersham Pharmacia Biotech). Transgenics in lanes 2, 4 and 7 represent single copy events. Transgenics in lanes 1 and 8 represent the same transformation event. Lane 10 is untransformed negative control.

The selfed progeny of heterozygous *tfdA* line, *tfdA* 1.32, with single copy insertion of the T-DNA segregated in 3 : 1 ratio at the level of seed growth (data not shown). The progenies with transgene showed proper hypocotyl elongation at all the concentrations of 2,4-D that were tested (Figure 2). These seedlings also rooted properly in media containing 2,4-D up to a concentration of 2.5 mg l^{-1}. However at the concentration of 5 mg l^{-1} of 2,4-D, root elongation of transgenic plants was delayed.

For determining the 2,4-D toxicity at the field level, T1 backcrossed progenies of representative single copy lines, viz. *tfdA* 1.4, *tfdA* 1.9, *tfdA* 1.32 and *tfdA* 2.1 were transplanted in the field along with non-transformed Varuna as a control. After 3–4 weeks of germination, seedlings were sprayed with different concentrations (10, 20, 50, 100, 200, 500 and 1000 mg l^{-1}) of herbicide 2,4-D (isopropyl ester; commercially available as Hammer 38, Pusti Agrochemical Pvt Ltd, India). The effect of 2,4-D spray on the survival of plants was analysed over a period of two weeks. Wild type plants and segregants without the *tfdA* gene were affected by 2,4-D levels as low as 10 mg l^{-1} and were completely killed at 50 mg l^{-1} and higher levels. Such sensitive plants showed wilting of leaves 24 h after the spray and were killed within 10–12 days. All the four transgenic lines tested in the current study were resistant to 10 times the concentration of 2,4-D (i.e. 500 mg l^{-1}), at which the wild-type plants get completely killed. Two lines *tfdA* 1.32 and *tfdA* 2.1 were resistant to 2,4-D herbicide even at a concentration of 1000 mg l^{-1} (data not shown). The backcrossed progeny of transgenic lines tested, segregated in 1 : 1 ratio of resistant to sensitive plants, thereby confirming the single copy status of the transgene (Table 2). Resistant plants in the segregating progeny of transgenic lines were confirmed for the presence of *tfdA* gene by tissue-PCR[19] using *tfdA* gene-specific primers (data not shown).

In the transgenic lines developed in this study, nosPr–*npt*II–ocspA cassette, used as the selectable marker for *in vitro* selection of T0 transformants, has been cloned within

Table 1. Transformation of *Brassica juncea* cv. Varuna using *tfdA* construct

No. of explants	No. of transformants	Transformation frequency (%)	No. of plants taken to field	No. of plants analysed for copy number	No. of single copy events
332	36	8.5	20	14	3
302	42	11.2	20	10	3

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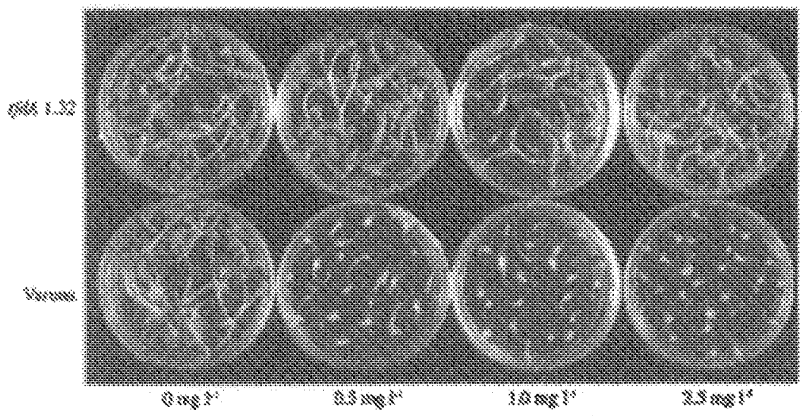


Figure 3. Seed germination of T1 selfed progeny of line *gfdA* 1.32 on MS medium supplemented with various concentrations of 2,4-D. Untransformed seeds of cultivar Varuna were also tested for germination as a control.

Table 2. 2,4-D spraying analysis on BC1 progenies of *gfdA* transgenic lines of *B. juncea*

Transgenic line	2,4-D at 50 mg l⁻¹ S	R	chi-square[a]
gfdA 1.4	34	31	0.14
gfdA 1.9	10	11	0.05
gfdA 1.32	30	25	0.55
gfdA 2.1	37	31	0.53
Varuna	78	9	ns

S, sensitive; R, resistant; ns, not tested; [a]chi-square was performed at 95% confidence limit ($P < 0.05$) to determine the goodness-of-fit.

the lox sites. After development of homozygous lines, the marker gene can be removed using lox/*cre* system of recombination[20]. The efficiency of lox/*cre* system for marker removal in mustard is being tested at present in the laboratory. Our limited field experiments have shown that *gfdA* gene could be used as an effective field level selection marker allowing the use of a cheaper and more readily available herbicide 2,4-D in place of a herbicide like 'Basta'. It would be interesting to check the efficacy of 2,4-D to control weeds in mustard including the root-parasite *Orobanche* spp. under field condition. With the availability of resistant lines, it will be also possible to see if the 2,4-D-resistant transgenics could be useful for low-till cultivation of mustard both for moisture conservation and for development of two-crop systems in the rain-fed and low-irrigation areas of North India.

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Validation of multi-channel scanning microwave radiometer on-board Oceansat-I

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Sea surface temperature (SST), sea surface wind speed (WS) and columnar water vapour (WV) derived from Multi-frequency Scanning Microwave Radiometer (MSMR) sensor on-board IRS-P4 (Oceansat-I) were validated against the *in situ* measurements from ship, moored buoy (MB), drifting buoy (DB) and autonomous weather station (AWS). About 1400 satellite *in situ* match-ups were used for the validation of SST and WS, while only 60 match-ups were available for the validation of WV. Therefore specific humidity, Q_s was used as a proxy for validating WV.

The drifting buoy SSTs showed good correlation with the satellite values ($r = 0.84$). The correlation of MB SSTs was better during night when the WS varied between 0 and 10 m/s. During the day, correlation peaked for higher wind speeds (> 10 m/s). MB ($r > 0.80$) was relatively better than AWS ($r \sim 0.70$) and ship ($r < 0.50$) for validating satellite-derived WS. Daytime winds exhibited better correlation with satellite values when measured from ocean platforms (MB and ship), but the winds measured from land-based platforms (AWS) were closer to satellite values during night-time. Q_s values consistently showed higher correlation with satellite values during night-time. The low root mean square deviation (RMSD) of DB SST (1.17°C) and MB WS (1.52 m s^{-1}) is within the achievable accuracy of the microwave sensor when validated with data collected over the tropical Indian Ocean. The RMSD of Q_s (1.81 g kg^{-1}), however, falls much beyond the attainable accuracy of the microwave sensor.

IRS-P4 (Oceansat-I) launched on 26 May 1999 carried an all-weather capable Multi-frequency Scanning Microwave Radiometer (MSMR) payload besides Ocean Colour Monitor (OCM). Details of these payloads are described elsewhere[1]. Channels of MSMR are suitable to retrieve sea surface temperature (SST), sea surface wind speed (WS), columnar water vapour (WV) and cloud liquid water on a global scale. The 6.6 GHz band, suitable for the retrieval of SST and WS (along with 10 GHz), was an improvement over the contemporary sensors (SSM/I, SSMR, etc.).

Weak microwave radiation in these channels makes the spatial resolution more coarse, but is still useful for supplementing high-resolution, cloud-sensitive AVHRR data[2]. Microwave radiometers can measure SST with an accuracy[3,4] of ± 1.5°C, which is much coarser than the one (± 0.5°C) attainable from thermal infrared sensors[5]. Wentz *et al.*[6] showed that passive microwave radiometers can measure WS with an accuracy of approximately 2 m s^{-1}, which is as good as the scatterometer and altimeter-derived WS. Retrieval accuracy of WV from Nimbus 5 microwave spectrometer was shown[7] to be 0.2 g cm^{-2} and it improved substantially to 0.07 g cm^{-2} when derived from SSM/I on-board DMSP satellite[8].

The objectives of this satellite validation exercise are to (a) identify the best frequency combinations to derive SST, WS and WV from satellite sensors by comparing with *in situ* data, (b) identify suitable *in situ* platform for validating satellite-derived SST, WS and WV, (c) study the role of WS in the satellite SST validation and (d) understand the accuracy of MSMR measurements.

The MSMR geophysical data product supplied by the National Remote Sensing Agency consists of 24 h data and has been generated on three different grid sizes, viz. 150 km (grid I), 75 km (grid II) and 50 km (grid III). Details of the grids are given elsewhere[1].

The sea truth data for SST, WS and WV were collected for two years from sensors mounted on various platforms

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Broad virus resistance in transgenic plants

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Viruses are significant threats to agricultural crops worldwide and the limited sources of natural resistance warrant the development of novel resistance sources. Several methods of transgenic protection have been successfully applied, including protein- and RNA-mediated approaches. Increased understanding of the molecular biology of virus infection is starting to bear fruit, enabling specific strategies to be designed for virus resistance in crops.

Over the years, resistance genes present in wild varieties of cultivated crops have been the main source of antiviral activity in breeding programs, but few have withstood the fast evolution of pathogens over an extended period of time in the field. Although direct interactions have not been found between resistance genes and their elicitors, a single amino acid mutation is often sufficient to change a pathogen from avirulent to virulent. Resistance in the field under high pathogen pressure allows rapidly mutating pathogens such as viruses to select such single amino acid mutants quickly. Moreover, plant RNA viruses in the field exist as swarms of point mutants (so-called quasi-species), which further enhances their ability to overcome resistance. Durability of plant virus resistance seems mostly to be dependent on the loss of fitness accompanying the required point mutation in the pathogen. The use of natural resistance genes as transgenes in other plant species has been demonstrated for some resistance genes, but other transgenes of a different nature give more options for transgenic virus resistance. Here some of the latest advances in breeding for resistance in transgenic crop plants are reviewed.

Application of pathogen-derived resistance

A variety of ways of generating transgenic virus resistance have been developed over the past decade. Most strategies originated from the pathogen-derived resistance (PDR) concept [1]. This involves the untimely expression of dysfunctional pathogen-derived products. Shortly after the concept was introduced, coat-protein-mediated-resistance (CPMR) against tobacco mosaic virus (TMV) was reported [2], followed by reports of protein-mediated resistance against a number of other economically important

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strategies, such as replicase-mediated resistance and movement-protein-mediated resistance [3]. In general, these strategies produced highly variable levels of resistance and the range of viruses targeted in individual experiments varied. In concert with this notion, and contradictory to the PDR concept, resistance levels often did not correlate with protein expression levels. The discovery that in many cases the expression of transgenic RNA, not the expression of viral proteins, was responsible for the observed resistance created unforeseen opportunities for resistance [4,5]. RNA-mediated virus resistance (RMVR) proved far more potent in inducing high levels of resistance, often displaying complete immunity to the inoculated virus or RNA, whereas protein-mediated resistance generally resulted in milder forms of resistance, such as delay in symptom development and decreased levels of virus titers. RNA-mediated resistance appeared to be highly sequence specific and thus limited to the virus from which the transgene was derived, whereas protein-mediated resistance had potential for broader resistance.

RNA silencing

A decade ago, Lindbo and co-workers [6] were the first to present evidence that the observed resistance mediated by transgenic RNA was related to the previously observed co-suppression phenomenon [7,8]. Later work in the plant virus community, notably by Baulcombe's group, revealed that RNA-mediated resistance was 'merely' preprogramming and thereby enhancing a previously unidentified antiviral strategy in plants, in a way that is best described as a potent genetic vaccination against viral sequences [9]. The discovery of a previously undisclosed class of plant-virus-encoded proteins capable of interfering with RNA silencing confirms this notion [10,11]. Since the discovery of what is now generally called RNA silencing in plants, the same specific RNA degradation mechanism has been identified in fungi, insects and vertebrates and is referred to as quelling or RNA interference [12]. No antiviral activity has been attributed to the process in fungi, insects or vertebrates, although this seems to be its main purpose in plants, together with emerging evidence for a function in developmental regulation [13]. Exploiting the increasing knowledge of RNA silencing from plant and animal models and the notion that it can be efficiently provoked by double-stranded RNA (dsRNA) sequences, Waterhouse's group [14] developed transgenes arranged in an inverted repeat (IR), expressing dsRNA sequences after transcription. They were able to increase the success rate of transgenic RNA-mediated resistance by an order of magnitude – further evidence that RMVR exploits RNA silencing. As a consequence, the range of applications for this strategy has broadened to include transformation-recalcitrant crops because the use of IR transgenes has shown that only a few successful transformation events are needed for

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An important drawback of using of RMVR in transgenic crops is the high level of sequence specificity. Viruses containing >10% nucleotide divergence are insensitive to this form of resistance, but with this level of divergence these viruses would generally be considered different species. Early attempts to overcome the problem by expressing transgenes of different viruses were successful [16]. However, the use of a large set of complete viral genes in an inverted repeat arrangement can lead to stability problems in the cloning and transformation processes with the size of the transgene being effectively trimmed to ~400 bp. However, significant gain can be made by linking virus-specific RNA sequences as small as 116 nt to green fluorescent protein RNA, which is used as a carrier [17].

Improving protein-mediated resistance

Compared with the success of RMVR, the recent development of protein-mediated resistance seems limited. Indeed, the reason for the success of RMVR could be the cause of that. Because protein-mediated resistance requires high levels of transgenic protein expression, its mRNA levels generally need to be high. However, like dsRNAs, high levels of transgenic mRNA trigger RNA silencing and thus RNA silencing seems to prevent high levels of protein expression in transgenic plants. To use protein-mediated resistance it therefore seems necessary to target early, essential processes that can be effectively disrupted by lower levels of transgenic protein expression.

In a recent report, Rudolph and co-workers [18] describe a novel approach that could add to the range of possibilities available for biotechnology to create novel types of broad virus resistance. Rather than using a full-size viral transgene in an attempt to generate protein-mediated resistance, they embraced a more literal interpretation of the PDR paradigm by first identifying an essential functional domain within a viral gene product. The target of this study, the nucleoprotein (N) of tomato spotted wilt virus (TSWV), is involved in almost all steps in the life cycle of the virus. These include several structural roles, such as protecting the viral RNA by cooperative binding, mediating the association with the polymerase to form infectious units, associating with viral movement proteins to facilitate the tubule-guided cell-to-cell movement process, and association with viral glycoproteins to form mature virus particles that can be transmitted by insects

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of the TSWV N gene and used a yeast two-hybrid screen to identify the capacity of these peptides for interacting with wild-type N protein, thereby attempting to identify the multimerization domain. This proved successful and, moreover, the authors succeeded in identifying a peptide with a conserved function by demonstrating that as well as binding to the N protein of TSWV, it can also bind to the N protein of related Tospoviruses tomato chlorotic spot virus (TCSV), groundnut ringspot virus (GRSV), chrysanthemum stem necrosis virus (CSNV) and impatiens necrotic spot virus (INSV). Transgenic plants were produced with the identified peptide fused to a carrier protein, β-glucuronidase (GUS). Although ineffective against the more distantly related INSV and less pronounced against TCSV, the expression of the aptamer fusion-protein resulted in broad *Tospovirus* resistance against TSWV, GRSV and CSNV.

Durability

Because of increasing public concern, mainly in Europe, regarding the use of transgenic crops, use of transgenic forms of resistance in the field has been limited. The durability of protein-mediated versus RNA-mediated resistance in the field and advantages of using transgenic forms of resistance over the use of natural resistance genes is therefore unknown, although some potential durability risks can be discerned.

The Achilles heel of protein-mediated resistance strategies, as with natural resistance genes, lies in the specific interactions with the viral target protein, which can be disrupted by a single amino acid change. Rapid adaptation by viruses in the field can only be countered by designing strategies (such as the aptamer strategy) to operate against essential domains of a protein that probably cannot be altered without inflicting serious reductions in virus fitness.

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The use of RNA-mediated resistance, apart from higher resistance levels, has the advantage that it is far less susceptible to variations in virus sequences. However the potential risk of disabling transgene-silencing-induced virus resistance by strong silencing suppressors of non-related viruses in mixed infections has been reported [20] and might indicate that a careful assessment of the viruses present in areas of interest is needed. Subsequently, RNA sequences of these viruses might have to be included in transformation constructs. Ultimately, the combination of RNA- and protein-mediated forms of transgenic resistance could prove to be high, broad and durable.

Acknowledgements

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EXPERIMENTAL PROTOCOLS

A Plant DNA Minipreparation: Version II

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The topic of this report is rapid microscale methods for isolation of plant DNA without the use of ultracentrifugation with CsCl. The DNA produced is of moderately high molecular weight and serves as a satisfactory substrate for most restriction endonucleases and is suitable for genomic blot analysis. In addition to the rapidity and convenience of minipreps which permit a large number of samples to be processed in just a few hours, the small amount of tissue required (less than 1.0 grams) allows for molecular analysis of plants at a very young stage. Miniprep DNA yields from leaf tissue of most species tested to date are typically 50-100 µg per gram tissue, greater than 50 kb, and remarkably uniform from sample to sample.

The first miniprep procedure we reported for maize DNA isolation (Dellaporta et al., *Maize Genetics Cooperation Newsletter*, 1983) was adapted from a procedure commonly used for yeast DNA preparation (Davis et al., 1980). Since this report, numerous personal communications have demonstrated that the miniprep procedure or a modification thereof, can be applied to most plant species tested. For example, the method has been successfully used on *Nicotiana tabacum*, *N. plumbaginifolium*, *N. sylvestris*, *Lycopersicon sp.*, *Amaranthus sp.*, *Glycine max*, *Petunia hybrida*. Several modifications have been applied by these investigators and in our own laboratory in order to extend the application of miniprep procedures to other plant species. The selection of a particular protocol depends to a large degree on the plant species used. However, the procedure reported here was selected to be suitable for most situations.

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Miniprep Procedure

1. Weigh 0.5 to 0.75 gm of leaf tissue, quick freeze in liquid nitrogen and grind to a fine powder in a 5 in. mortar and pestle. Transfer powder with liquid nitrogen into a 30 ml Oak Ridge tube.

It is imperative not to let the tissue thaw once frozen until buffer is added and not to cap the tubes while nitrogen is evaporating.

2. Add 15 ml of Extraction Buffer (EB): 100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 10 mM mercaptoethanol.

For maximum DNA yields, the cells are further broken by grinding the mixture at a low setting (about 3) with a Polytron (Brinkmann Instruments, Inc.). However, this step is optional.

3. Add 1.0 ml of 20% SDS, mix thoroughly by vigorous shaking, and incubate tubes at 65°C for 10 min.

4. Add 5.0 ml 5 M potassium acetate. Shake tube vigorously and incubate 0° for 20 min.

Most proteins and polysaccharides are removed as a complex with the insoluble potassium dodecyl sulfate precipitate.

5. Spin tubes at 25,000 × *g* for 20 min. Pour supernatant through a Miracloth filter (Calbiochem) into a clean 50 ml tube containing 10 ml isopropanol. Mix and incubate tubes at −20° for 30 min.

6. Pellet DNA at 20,000 × *g* for 15 min. Gently pour off supernatant and lightly dry pellets by inverting the tubes on paper towels for 10 min.

7. Redissolve DNA pellets with 0.7 ml of 50 mM Tris, 10 mM EDTA, pH 8. Transfer the solution to an Eppendorf tube. Spin the tubes in a microfuge for 10 min to remove insoluble debris.

8. Transfer the supernatant to a new Eppendorf tube and add 75 µl 3M sodium acetate and 500 µl isopropanol. Mix well and pellet the clot of DNA for 30 sec in a microfuge. Wash pellet with 80% ethanol, dry, and redissolve in 100 µl 10 mM Tris, 1 mM EDTA, pH 8.

Precipitation from 0.5 M sodium acetate using relatively small amounts of isopropanol (about 0.6 volumes) has been reported to separate high molecular DNA from polysaccharides (Marmur, 1961). The sodium acetate also yields a tight fibrous precipitate that is easily washed and dried. The DNA will dissolve readily if allowed to rehydrate at 4° for one hour followed by light vortexing.

Possible Modifications

The following modifications can be applied to the miniprep procedure if problems with nucleases or contaminants that prevent restriction digest of the DNA are encountered. This procedure is also preferred for "difficult" species such as soybean.

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1. Tissue is frozen in liquid nitrogen and freeze dried. The lyophilized tissue is ground to a fine powder with a mortar and pestle.

2. Follow steps 2 through 7 of protocol 1.

3. Add 50 µl 3M NaOAc and 100 µl 1% CTAB (Cetyl trimethylammonium bromide), which will precipitate nucleic acids. Pellet the CTAB precipitate for 30 sec. in the microfuge and wash the pellet with 70% ethanol.

4. Redissolve the pellet in ~400 µl TE. Ethanol precipitate the DNA with 50 µl 3M NaOAc and 1 ml ethanol.

The CTAB-DNA precipitate may not entirely redissolve but continue with the ethanol precipitation.

5. Repeat step 4. The pellet should entirely redissolve and the second ethanol precipitation should remove the residual CTAB leaving the DNA in the sodium form.

6. Finally redissolve the dry DNA pellet in 10 mM Tris, 1mM EDTA pH8 per gm starting material.

Minipreps can be stored for several months without evidence of degradation and can be cut with a variety of restriction enzymes and ligated without further purification. We find that 10.0 µl of miniprep DNA is sufficient for a single 8 mm lane in an agarose gel which is to be used for filter hybridization with single-copy probes. Heat-treated RNAase must be added to the restriction reaction to digest contaminating RNA in each prep. Hence, a typical reaction would contain the following:

Miniprep DNA	10.0µl
10X Restriction Buffer	3.0µl
0.5 mg/ml RNAase	2.0µl
Eco RI	8.0 units
dH20 to 30 µl	

Digestion is usually complete after 3 hours at 37°. Occasionally, minipreps are difficult to digest with certain enzymes. This problem can be overcome by adding 5.0 µl of 0.1 M spermidine to the entire miniprep before digestion (see Focus 4(3) 12, 1982).

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Opposition by

Bayer CropScience NV

against

European Patent **EP-B-2 308 976**
granted in the name of Dow AgroSciences LLC.

NOTICE OF OPPOSITION

1. Introduction and Requests

Opposition against European Patent n° EP 2 308 976, granted in the name of Dow AgroSciences LLC, entitled "NOVEL HERBICIDE RESISTANCE GENE", is hereby filed by Bayer CropScience NV, J.E. Mommaertslaan 14, BE-1831 Diegem, Belgium.

It is requested that the patent as granted is revoked under Articles 100(a), 100(b) and Art 100(c) EPC.

Oral proceedings are also requested under Article 116 EPC in the event that the Opposition Division does not intend to revoke the patent on the basis of the written submissions.

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2. Cited Documents

In support of the opposition, Opponent cites the following documents:

D1: Autoradiographs from hybridization experiments between AAD-1 and tfdA nucleic acids

D2: Streber, Timmis and Zenk, 1987. J. Bacteriology 169(7): 2950-2955

D3: Fukumori and Hausinger 1993 J. Bacteriology 175(7): 2083-2086

D4: amino acid sequence alignment between TFDA and AAD1 protein

D5: Hulse, Ellis and Henderson 1978 J. Biol. Chemistry 253: 1654-1659

D6: Eichorn, van der Ploeg, Kertesz and Leisinger 1997 J. Biol. Chemistry 272 (37): 23031-23036

D7: priority document USSN 60/567,052

D8: Streber and Willmitzer 1989 Biotechnology 7: 811-816

D9: Lyon, Llewellyn, Huppatz, Dennis and Peacock 1989 Plant Molecular Biology 13 : 533-540

D10: Lyon, Cousins, Llewellyn and Dennis 1993 Transgenic Research 2 : 162-169

D11: Bayley, Trolinder, Ray, Morgan, Quisenberry and Ow 1992 Theor. Appl. Genet 83:645-649

D12 : Bisht, Burma and Pental 2004 Current Science 87(3) : 367-370

D13 : alignment of the nucleotide sequence of SEQ ID No. 3 of the contested patent with the nucleotide sequence of entry AF516751 (Rhodoferax sp P230 rdpA gene).

D14: AF516751 nucleotide sequence, version of Jun 26, 2003

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D15: Schleinitz, Kleinsteuber, Vallaeys and Babel 2004 Applied and Environmental Microbiology 70(9): 5357-5365

D16: alignment of the nucleotide sequence of SEQ ID No. 3 of the contested patent with the nucleotide sequence of entry AJ628859 (Sphingomonas herbicidovorans tnpA gene)

D17: AJ628859 nucleotide sequence version of Mar 3 2004.

D18: WO 97/13402

D19: WO87/05629

D20: WO 03/013224

D21: US 5,463,175

D22: US2003/0135879

D23: WO96/33270

D24: Prins, 2003 Trends in Biotechnology Vol 21 No. 9 373-375

D25: Dellaporta, Wood and Hicks, 1983 Plant Molecular Biology Reporter 1 (4) pages 19-21

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3. Interpretation of the claims as granted

3.1 *Terms of the claims*

In the below analysis of the claims with the patentability requirements, the following term in the claims has been construed in accordance with the teaching of the application, or in absence thereof, been given its broadest meaning in accordance with established case-law (T686/96; T79/96).

3.1.1 "hybridizing under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID No. 9 ".

The application as filed, page 30, paragraph 127, provides the following guidance: "*As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants*".

The application further refers to standard methods as e.g. defined by Maniatis et al. 1982 to perform hybridization of immobilized DNA on Southern blots with 32P-labeled gene-specific probes. In paragraph 131, spanning pages 30 and 31, examples of salt conditions are provided to influence the stringency in hybridizations when using labeled DNA fragments longer than 70 bases in length, including low, moderate or high stringent conditions.

Similarly, paragraph 124 on page 29 provides guidance on hybridization. Specifically, it is indicated that hybridization can be followed by subsequent washes under conditions of low, moderate or high stringency, which are achieved by using first washes with 2xSSC/01% SDS for 15 minutes at room temperature (low); subsequently with 0.1%SSC/0.1%SDS at room temperature (moderate) and then with 0.1%SSC/0.1%SDS for 30 minutes at 55°C. The application also teaches that "higher stringency can be achieved by lowering the salt concentration and/or by raising the temperature".

It is important to realize that the claims merely require "hybridization" and "stringent" conditions, which is not to be equated with "specific hybridization" and "high stringent" conditions.

Document D1 consists of three autoradiographs, resulting from a hybridization experiment performed by an independent contracting lab.

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Using a 0.8% TBE agarose gel, the following samples and controls were loaded:

- varying amounts (1000ng, 500ng, 250ng, 100ng, 50ng, and 10ng) of target A (*aad1*) PCR product
- varying amounts (500ng, 1000ng, 250ng, 100ng, 50ng, and 10ng) of target B (*aad12*) PCR product,
- two dilutions (1ng and 0.25ng) of the positive control tfdA PCR product DNA
- 500ng of the negative control pBR322 DNA (PCR product~850bp)
- 10µg of the negative control salmon sperm gDNA X Bam H1 digested
- 10µg of the negative control salmon sperm gDNA X Eco R1 digested
- 10µg of the negative control salmon sperm gDNA X Hind III

Invitrogen 1Kb+ DNA ladder was used for sizing

Standard S. blot transfer procedures for the turbo blotter (descending transfer) were employed.

The nylon filter was UV linked and prehybridized (10mM EDTA, 6 X SSC, 0.5% SDS, 10% formamide, 5 X Denhardt's solution, and 100ug/ml denatured sheared salmon sperm DNA) for 4 hours at 42 degrees C. with shaking.

The filter was hybridized using 20 ml of the same buffer used for the prehybridization solution and 1000ng of T4 PNK 5' end labeled 32P tfdA DNA (total of 8,000,000dpm per 20ml) at 42 degrees C. for 24 hours.

The filter was rinsed at room temperature in 3 X SSC + 0.5% SDS for 5 minutes, followed by 2 rinses at room temperature (15 minutes each) in 3 X SSC + 0.1% SDS.

The **first wash** (3 changes) was carried out for **60 minutes** using **3 x SSC+ 0.1% SDS, at 35° C.**
The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1a)

The **second wash** (3 changes) was carried out for **2 hours** using **2 X SSC + 0.1% SDS at 68° C.**
The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1b)

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The **third wash** (3 changes) was carried out for **40 minutes** using **0.1 X SSC + 0.1% SDS at 68° C**. The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1c).

The autoradiograph obtained after **first wash (low stringency; D1a)** showed negative control background with very strong positive control and target sample signal. The autoradiograph obtained after the **second wash (moderate stringency; D1b)** demonstrates that this moderate stringency wash removed nearly all of the negative control background and left strong positive control and target sample signal. The autoradiograph obtained after the **third wash (high stringency; D1c)** demonstrates that the third wash totally removed the negative control signal and left very strong positive control signal and good target sample signal even down to 10ng of both target DNAs.

"Target A (aad1)" corresponds to a nucleic acid encoding a protein of SEQ ID No. 9.

"TfdA" corresponds to a nucleic acid having the nucleotide sequence of tfdA gene of *Ralstonia eutropha* (Streber et al. 1987; D2)

The application at page 3, paragraph 11 admits that "*TfdA catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction*". D3 (Fukumori and Hausinger) confirms the same. TfdA thus encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

The proteins encoded by TfdA gene and by the aad1 gene have 32% amino acid sequence identity (D4).

Even under stringent hybridization conditions, the nucleotide sequence encoding SEQ ID No. 9 specifically hybridizes to a nucleic acid encoding a protein having 2,4 D enzymatic degrading activity, whereby the nucleic acid was known from the art.

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4. Added Subject Matter (Articles 76(1), 123(2) EPC and 100 (c))

The subject matter of the granted claims goes beyond the disclosure of the application as filed. The claims thus contravene the requirements of Article 123(2) EPC. It should be noted that the current divisional application as filed appears to be an exact copy of the parental application as filed. At that time no claims were filed, but the originally filed claims were copied as items in the description. Only on January 27, 2011, a set of claims to be pursued in the current application was filed. Accordingly, any subject matter identified as not being present in the divisional application will equally be subject matter extending beyond the scope of the originally filed application (Art 76(1) EPC and vice versa).

4.1 *Claims 1, 17 and 18 contain subject matter not disclosed in the parent application as filed*

In his letter of January 27, 2011, the Patentee indicates originally filed claim 5 as basis for claim 1, together with paragraphs 112, 118 and 124-131 as support for hybridization conditions.

Claim 1 as filed on January 27, 2011 and granted in unamended form, reads:

> *A transgenic plant comprising an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID No: 9, where said plant expresses said polynucleotide and produces said protein.*

Originally filed claim 5 reads:

> *A transgenic plant comprising a plurality of cells according to claim 1, wherein expression of said polynucleotide renders said cells tolerant to an aryloxyalkanoate herbicide.*

Originally filed claim 1 reads:

> *A transgenic plant cell comprising* ***a polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO: 9 or a variant of SEQ ID NO: 9 wherein said variant has aryloxyalkanoate dioxygenase activity, at least one amino acid deletion or conserved substitution, and at least 95% sequence identity with SEQ ID NO: 9*** *(emphasis added).*

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In other words, the transgenic plant of claim 5 as originally filed was limited to plants expressing AAD-1 or a highly related variant thereof having a particular enzymatic activity. In contrast, the transgenic plant of granted claim 1 extends to transgenic plants comprising any polynucleotide hybridizing to any nucleotide molecule that encodes AAD-1 of any enzymatic activity which may degrade phenoxy auxin herbicides or aryloxyphenoxypropionate herbicide.

Paragraphs 112, 118 and 124-131 merely describe hybridization conditions and indicate that a class of proteins can be defined by hybridization properties of the encoding polynucleotides, but does not make reference to the specific combination of characteristics now claimed.

Given the wide range of nucleic acids that can hybridize even under stringent conditions to the mentioned nucleotide sequence (see above), it will be clear that subject matter has been included in claim 1 which is not unambiguously and directly derivable from the application as originally filed and thus contravenes the requirements of Art 76(1).

The argumentation applies mutatis mutandis to the subject matter of independent claims 17 and 18 which refer to the plant of claim 1.

In particular, claim 17 refers to an "asexual propagate" of the plant of claim 1. This term is only used once in the context of originally filed claim 59 referring to the plant of originally claim 5.

As basis for claim 18, directed towards ground tissue from transgenic maize plants comprising a polynucleotide hybridizing to any nucleotide molecule that encodes AAD-1 of any enzymatic activity which may degrade phenoxy auxin herbicides or aryloxyphenoxypropionate herbicide, Patentee referred to specific Examples wherein, during the DNA analysis of transgenic tissue or plants comprising a particular version of AAD1 encoding nucleotide sequence, particular tissue has been grinded, either from maize calli, (not plants) (paragraph 320), rice plantlets (paragraph 478) or canola plants. The latter two clearly do not provide support for ground tissue from a corn plant, but also the first cited passage is directed to a particular combination of features, which does not allow to derive the further combination with the overbroad generalized subject matter of claim 1.

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4.2 ***Claims 2-16 contain subject matter not disclosed in the parent application as filed***

Claims 2-16 are dependent upon claim 1. These claims therefore also contravene the requirements of Art 76(1). However, several dependent claims make further new combinations that cannot be unambiguously and directly derived from the parent application as filed.

E.g. claim 14 claims plants of claim 1 further comprising an insect-resistance gene derived from a number of listed organisms while claim 15 claims plants of claim 1 further comprising a gene for a selected agronomic trait. The only basis to be found for these broad terms are in originally filed claims 61 and 62. However, both refer to the plant as claimed in original claim 5, i.e. transgenic plants with a much more circumscribed definition of the nucleotide sequence included in those transgenic plants.

Claim 3 claims plants further comprising a glufosinate and a glyphosate tolerance trait. According to the Patentee, support for the subject matter of claim 3 can be found in paragraphs 16, 82, 98, 344, Table 25 and 346. However, paragraph 16 mentions only that the invention includes "plants producing one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including but not limited to glyphosate, imidazolinone and glufosinate resistance genes". That is not a specific disclosure of plants further comprising only two specifically chosen tolerance genes from the list. Similarly, paragraph 82 lists even more herbicide tolerance genes which may be stacked, but does not single out specifically the further combination with glyphosate and glufosinate tolerance genes. Idem for paragraph 98. The other cited passages relate to Example 8, describing corn(maize) plants with specifically the AAD1(v3) transgene stacked with a glyphosate and glufosinate tolerance gene. However, this passage cannot provide the broad support required for current claim 3, claiming any plant with the combination of glufosinate and glyphosate tolerance traits with a transgene containing any polynucleotide hybridizing to any nucleotide molecule that encodes AAD-1 of any enzymatic activity which may degrade phenoxy auxin herbicides or aryloxyphenoxypropionate herbicide.

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4.3 ***Claims 1 -18 contain subject matter not disclosed in the divisional application as filed.***

As indicated above, any subject matter not present in the parent application as filed is necessarily also not present in the divisional application as filed, and the deficiencies identified above under Art 76(1) are also deficiencies under Art 123(2).

Priority entitlement (Article 87 EPC)

As will be demonstrated below, the claims as granted are not entitled to the benefit of the priority date of 30 April 2004 (filing date of US60/567,052 D7). Consequently, the relevant date for determining the prior art base is the international filing date, i.e. May 2, 2005.

Comparing the application as filed to the priority document, it will be immediately apparent that the priority application contains less teaching than the parent and divisional application as filed. In particular, no claims as originally filed in the parent application (and included as items in the divisional application) are included in the priority document, as well as a number of Sequences, Figures and Examples are missing from the priority document.

Decision of the Enlarged Board of Appeal G2/98 has confirmed that an invention is only entitled to priority when the priority application clearly and unambiguously discloses that invention.

As set out above in connection with Art 100 (c), Art 76 and Art 123(2), the terms of the claims as granted cannot even be derived clearly and unambiguously from the parent or divisional application.

As the priority application contains even less teaching than the parent application or divisional application as filed, the subject matter of the claims can also not be derived clearly and unambiguously from the priority application as filed. The subject matter of these claims is therefore not entitled to any of the claimed priorities in accordance with G2/98.

However, there are additional reasons why the claims are not entitled the benefit of the earlier priority dates.

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As mentioned above, there are no claims included in the priority application. While some of the terms used in the current claims indeed can be found at various places in the application, there is no specific, clear and unambiguous disclosure of the combination of such features. Moreover, there are general terms which are only used once in the application as originally filed, exactly in the claims as filed, and therefore have no disclosure in the priority application.

E.g. the concept of " a protein that enzymatically degrades a herbicide selected from the group of a phenoxy auxin and an aryloxyphenoxyproprionate" as used in claim 1 can only be found in originally filed claim 47 in the context of an isolated polynucleotide sequence hybridizing with any one of three exactly defined nucleotide sequences encoding AAD1, and has no counterpart in the priority application nor can it be unambiguously derived therefrom. Claim 1 is thus not entitled to priority.

Claims 2-18 are dependent or refer to claim 1, and thus suffer from the same deficiency.

Additionally, the generic terms of claims 14 (insect resistance gene derived from listed organisms), 15 (gene providing specifically listed agronomic traits) and 17 (asexual propagate) can only be found in claims 61, 62 and 59 in the originally filed application and have no counterpart in the priority application.

In conclusion, none of the subject matter of the claims can be derived clearly and unambiguously from the disclosure of the priority application.

The relevant date to establish the prior art base for all claims is therefore the international filing date of May 2, 2005. Consequently, documents D12 and D15 are prior art within the meaning of Art 54(2) EPC.

5 Lack of Novelty (Articles 54 EPC and 100(a))

The subject-matter of several of the granted claims lacks novelty over the documents which will be discussed in detail in the following sections.

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5.1 *Claims 1, 9, 12, and 17 lack novelty over D8*

D8 is a first document describing transgenic plants expressing a bacterial detoxifying enzyme which plants are resistant to 2,4-D herbicide. D8 also describes isolated polynucleotides based on a tfdA gene from the soil bacterium *Alcaligenes eutrophus*, now renamed as *Ralstonia eutropha*, JM134 under control of a CaMV 35S promoter (see page 811, right column, section entitled "Construction of plasmids for constitutive and light induced DPAM expression in plants"). Transgenic plants were grown in the greenhouse and sprayed with 2,4-D (page 813, right column, first paragraph).

5.1.1 *Claim 1 lacks novelty over D8.*

As indicated above (section 3.1.1.) the *tfda* coding region (D2; reference 9 in D8) hybridizes under stringent conditions with a nucleic acid encoding a protein of SEQ ID No. 9 (and thus necessarily also with the complement). *tfdA* has 2,4 D degrading enzymatic activity (application as filed, page 3, paragraph 11). 2,4D herbicide has been sprayed on the transgenic plants. Plants were tolerant to application of up to 10 kg/ha 2-D-NA (page 813, right column, first paragraph). The tolerance phenotype indicates that the plants express the polynucleotide and produce the protein.

D8 thus anticipates claim 1 by disclosing all features of the subject matter of claim 1.

5.1.2 *Claim 9 lacks novelty over D8.*

Claim 9 further specifies the subject matter of claim 1 by requiring that the herbicide is a 2,4-D herbicide.

D8 discloses tolerance of the transgenic plants to 2,4 D.

Claim 9 is thus anticipated by D8.

5.1.3 *Claim 12 lacks novelty over D8.*

Claim 11 further defines the subject matter of claim 1 by specifying that the herbicide is selected from a group of herbicides, including 2,4-D.

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D8 discloses tolerance of the transgenic plants to 2,4 D.

D8 therefore discloses a transgenic plant as claimed in claim 12.

5.1.4 *Claim 17 lacks novelty over D8.*

Claim 17 is drawn to a plant part, a seed, a progeny, tissue or asexual propagate of the plant of claim 1.

D8 (page 814, left column first paragraph) discloses progeny and seeds of the transgenic plants, germinating into plants on 2,4-D medium, 20 of which were sprayed with 2,4-D. Southern analysis confirmed the presence of the transgene in the progeny plants.

Claim 17 is anticipated by D8.

5.2 *Claims 1 , 9, 12 and 17 lack novelty over D9*

D9 is another document describing cloning of a tfdA gene under control of a CaMV35S promoter and expressing it in plants, leading to 2,4 D tolerance. D9 discloses that the tfda region is operably linked to a CaMV35S promoter (page 535, left column, line 4), which is a promoter functional in plant cells. Leaf discs of the transgenic plants were assayed for their ability to shoot in the presence of 2,4-D herbicide (page 535, left column). Progeny of transgenic plants were germinated and grown for 4 to 8 weeks in soil, prior to spraying with 2,4 D herbicide (pages 538 left column, second section). 2,4D- tolerance was observed in transgenic plants and their progeny. D9 anticipates claims 1, 9, 12 and 17 for the same reasons as explained under 5.1.

5.3 *Claims 1, 9, 12 and 17 lack novelty over D10*

D10 is yet another publication wherein the tfdA gene of *A. eutrophus* JMP34 is cloned under the control of the CaMV 35S promoter (page 163, left column, second paragraph, lines 1 to 4). Transgenic cotton plants comprising this transgene are generated and sprayed with 2,4-D herbicide (page 167, left column, last paragraph; right column, end of the first paragraph). 2,4-D tolerant plants were obtained (see page 166, Figure 3 and page 168, Figure 4).

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D10 thus anticipates claims 1 , 9, 12 and 17 for the same reasons as explained under 5.1.

5.4 *Claims 1, 9, 12 and 17 lack novelty over D11*

D11 is a publication wherein the tfdA gene of *A. eutrophus* is cloned under the control of the CaMV 35S promoter (page 645 right column, last paragraph; page 646, left column, lines 14 to 16). Transgenic tobacco and cotton plants were generated and sprayed with 2,4-D herbicide (see page 648, Fig 2; page 648 right column paragraph preceding the concluding remarks). Transgenic plants were tolerant to the herbicide (Fig 2 and Fig 4). Progeny plants were also scored for herbicide tolerance (page 648 right column, last paragraph of the section "Transgenic cotton plants").

D11 therefore also anticipates claims 1, 9, 12 and 17 as elaborated under 5.1.

5.5 *Claims 1, 9, 12 and 17 lack novelty over D12*

D12 is the last publication cited wherein the tfdA gene of *A. eutrophus* is cloned under the control of the CaMV 35S promoter (page 367 right column, third paragraph). Transgenic mustard plants were generated and sprayed in field trials with 2,4-D herbicide (page 368, right column, third paragraph and Table 2). Transgenic mustard plants and their progeny were tolerant to 2,4 D herbicide application (Fig 2, Table 2).

D12 therefore also anticipates claims 1, 9, 12 and 17 as elaborated under 5.1.

5.6 *Conclusion*

Claims 1 , 9, 12 and 17 are anticipated by one or more of documents D8, D9, D10, D11 or D12.

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6 Inventive step (Articles 56 EPC and 100(b))

6.1 Claims which are strictly speaking considered novel over any one of D8 to D12 lack an inventive step.

Claims 1 , 9, 12 and 17 are anticipated by one or more of documents D8 to D12. For the remaining claims, Opponent submits they lack an inventive step over the prior art.

6.1.1 *Claims 2 to 7, 11 and 13 lack an inventive step over D8 (or any one of D9, D10, D11, D12) in combination with any one of D19, D20, D21 or D22.*

Claims 2 to 7, 11 and 13 further define the transgenic plant of claim 1 by specifying that the plants may comprise other herbicide tolerance genes, particularly glufosinate tolerance genes, glyphosate tolerance genes, dicamba tolerance genes and genes conferring tolerance to ALS inhibitor herbicides and that tolerance to the corresponding herbicides may also be provided.

Documents D8 to D12 do not disclose such additional herbicide genes. However, the application does not claim any interaction between the presence of the phenoxy auxin herbicide or aryloxyphenoxypropionate herbicide degrading transgene and the other herbicide tolerance genes. The problems can thus be separated and merely amounts to the provision of plants with these other tolerance genes in addition to the problem of providing plants with the transgene as specified in claim 1.

It will be clear that these other tolerance genes, and their application for generating herbicide tolerant plants to be used in weed control programs is well know from the prior art, including D19 (glufosinate tolerance), D20 (glufosinate tolerance), D21 (glyphosate tolerance), D22 (dicamba tolerance) and D23 (ALS inhibitor tolerance).

No inventive step can be derived from combining the transgene of claim 1 with the other herbicide tolerance genes.

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6.1.2 *Claim 8 lacks an inventive step over D8 (or any one of D9, D10, D11, D12)*

Claim 8 merely defines that the transgenic plant of claim 1 is a maize plant. No specific advantage is alleged for generating transgenic maize plants, rather than transgenic cotton, mustard, or tobacco plants and the choice of a particular plant cannot provide an inventive step.

6.1.3 *Claim 10 lacks an inventive step over D8 (or any one of D9, D10, D11, D12) in combination with D15*

D8 discloses a method of weed control using transgenic plants comprising a tfdA encoding transgene, rather than the rdpA of SEQ ID No. 9, disclosed in the contested patent.

The technical problem can thus be formulated as providing transgenic plants with alternative phenoxy auxin or aryloxyphenoxypropionate herbicide degrading enzyme encoding transgenes.

D15 discloses such coding regions involved in phenoxy auxin herbicide degradation. In fact, D15 discloses a coding region that is identical to SEQ ID No. 3 (except for an ambiguous Y nucleotide instead of C, see D13 and D14) and the encoded protein (SEQ ID No. 9) is identical.

A person skilled in the art would have substituted the tfdA gene in the polynucleotide of e.g. D8 for the coding region encoding the protein with enzymatic herbicide degrading capacity as disclosed in D15 to arrive at the claimed subject matter with reasonable expectation of success in view of the successes reported in any one of D8 to D12.

No inventive skill is needed to arrive at the subject matter of claim 10.

6.1.4 *Claims 14 and 15 lack an inventive step over D8 (or any one of D9, D10, D11, D12) in combination with D18 or D24, respectively.*

Claims 14 and 15 further define the transgenic plant of claim 1 by specifying that the plants may also comprise other transgenes conferring interesting traits such as insect-resistance genes (claim 14) or genes providing an agronomic trait, including virus resistance (claim 15).

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Documents D8 to D12 do not disclose such additional transgenes. However, the application does not claim any interaction between the presence of the phenoxy auxin herbicide or aryloxyphenoxypropionate herbicide degrading transgene and the other transgenes. The problem can thus be separated into subproblems and merely amounts to the provision of plants with these other transgenes in addition to the problem of providing plants with the transgene as specified in claim 1.

It will be clear that these other transgenes, and their application in agronomy are well known from the prior art, including D18 (BT insect-resistance genes) or D24 (virus resistance).

No inventive step can be derived from combining the transgene of claim 1 with other interesting transgenes known in the art.

6.1.5 *Claim 18 lacks an inventive step over D8 (or any one of D9, D10, D11, D12)*

Claim 18 is directed towards ground tissue from the plant of claim 8.

Documents D8 to D12 describe the analysis of DNA from transgenic plants comprising a transgene as described in claim 1. See e.g. D8, page 815, right column, "hybridization experiments"). Isolation of DNA commonly involves a step of grinding of tissues (as indicated in the contested patent). In particular, the method by Dellaporte used in D8 (submitted herewith as D25) uses a grinding step (see page 20 lines 2-3 of D25).

D8 to D12 therefore describe ground tissue from a plant according to claim 1. The difference with the subject matter of claim 18 is that the ground tissue is from a maize plant.

No specific advantage is alleged for generating transgenic maize plants, rather than transgenic cotton, mustard, or tobacco plants and the choice of a particular plant cannot provide an inventive step

7 Sufficiency of Disclosure (Articles 83 EPC and 100(b))

The invention as claimed in the patent as granted is insufficiently disclosed and therefore contravenes the requirements of Article 83 EPC.

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The application provides support and disclosure only for a very limited number of methods and polynucleotides i.e. those having SEQ ID No. 3-5 which encode a polypeptide having SEQ ID No. 9 and transgenic plants comprising such polynucleotides.

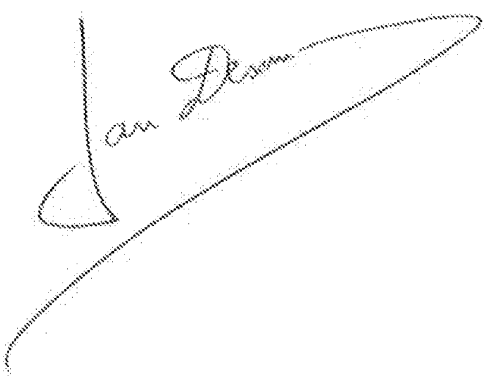
As demonstrated above, the definition of the claimed polynucleotides by virtue of capacity to hybridize under stringent conditions, still encompasses a large genus of polynucleotides including polynucleotides encoding a protein having as little as 32% amino acid sequence identity, and is thus not congruent with the limited disclosure by the application.

Remark

Opponent further remarks that claim 10 of the current patent claims the same subject matter as claim 5 of EP 1 740 039 granted on the parent application. It is an accepted principle in most patent systems that two patents cannot be granted to the same applicant for one invention. The Enlarged Board of Appeal has accepted obiter dictum that the principle of the prohibition on double patenting is based on the notion that an applicant has no legitimate interest in proceedings leading to the grant of a second patent for the same subject-matter if he already possesses one granted patent for that subject-matter (see G 1/05, and G 1/06).

Conclusion

The subject matter of the claims as granted does not meet the requirements of the EPC. The patent should be revoked in its entirety.



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Opposition by

Bayer CropScience N.V.

against

European Patent EP-B-2 298 901
granted in the name of Dow AgroSciences LLC.

NOTICE OF OPPOSITION

1. Introduction and Requests

Opposition against European Patent n° EP 2 298 901, granted in the name of Dow AgroSciences LLC, entitled "NOVEL HERBICIDE RESISTANCE GENES", is hereby filed by Bayer CropScience N.V., J.E. Mommaertslaan 14, BE-1831 Diegem, Belgium.

It is requested that the patent as granted is revoked under Articles 100(a), 100(b) and Art 100(c) EPC.

Oral proceedings are also requested under Article 116 EPC in the event that the Opposition Division does not intend to revoke the patent on the basis of the written submissions.

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2. Cited Documents

In support of the opposition, Opponent cites the following documents:

D1: Autoradiographs from hybridization experiments between AAD-1 and tfdA nucleic acids

D2: Streber, Timmis and Zenk, 1987. J. Bacteriology 169(7): 2950-2955

D3: Fukumori and Hausinger 1993 J. Bacteriology 175(7): 2083-2086

D4: amino acid sequence alignment between TFDA and AAD1 protein

D5: Hulse, Ellis and Henderson 1978 J. Biol. Chemistry 253: 1654-1659

D6: Eichorn, van der Ploeg, Kertesz and Leisinger 1997 J. Biol. Chemistry 272 (37): 23031-23036

D7: priority document USSN 60/567,052

D8: Streber and Willmitzer 1989 Biotechnology 7: 811-816

D9: Lyon, Llewellyn, Huppatz, Dennis and Peacock 1989 Plant Molecular Biology 13 : 533-540

D10: Lyon, Cousins, Llewellyn and Dennis 1993 Transgenic Research 2 : 162-169

D11: Bayley, Trolinder, Ray, Morgan, Quisenberry and Ow 1992 Theor. Appl. Genet 83:645-649

D12 : Bisht, Burma and Pental 2004 Current Science 87(3) : 367-370

D13 : alignment of the nucleotide sequence of SEQ ID No. 3 of the contested patent with the nucleotide sequence of entry AF516751 (Rhodoferax sp P230 rdpA gene).

D14: AF516751 nucleotide sequence, version of Jun 26, 2003

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D15: Schleinitz, Kleinsteuber, Vallaeys and Babel 2004 Applied and Environmental Microbiology 70(9): 5357-5365

D16: alignment of the nucleotide sequence of SEQ ID No. 3 of the contested patent with the nucleotide sequence of entry AJ628859 (Sphingomonas herbicidovorans tmpA gene)

D17: AJ628859 nucleotide sequence version of Mar 3 2004.

D18: WO 97/13402

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3. Interpretation of the claims as granted

3.1 *Terms of the claims*

In the below analysis of the claims with the patentability requirements, the following term in the claims has been construed in accordance with the teaching of the application, or in absence thereof, been given its broadest meaning in accordance with established case-law (T686/96; T79/96).

3.1.1 "hybridizing under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID No. 5, SEQ ID No. 3 and SEQ ID No. 4".

The application as filed, page 30, paragraph 127, provides the following guidance: "*As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants*".

The application further refers to standard methods as e.g. defined by Maniatis et al. 1982 to perform hybridization of immobilized DNA on Southern blots with 32P-labeled gene-specific probes. In paragraph 131, spanning pages 30 and 31, examples of salt conditions are provided to influence the stringency in hybridizations when using labeled DNA fragments longer than 70 bases in length, including low, moderate or high stringent conditions.

Similarly, paragraph 124 on page 29 provides guidance on hybridization. Specifically, it is indicated that hybridization can be followed by subsequent washes under conditions of low, moderate or high stringency, which are achieved by using first washes with 2xSSC/01% SDS for 15 minutes at room temperature (low); subsequently with 0.1%SSC/0.1%SDS at room temperature(moderate) and then with 0.1%SSC/0.1%SDS for 30 minutes at 55°C. The application also teaches that "higher stringency can be achieved by lowering the salt concentration and/or by raising the temperature.

It is important to realize that the claims merely require "hybridization" and "stringent" conditions, which is not to be equated with "specific hybridization" and "high stringent" conditions.

Document D1 consists of three autoradiographs, resulting from a hybridization experiment performed by an independent contracting lab.

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Using a 0.8% TBE agarose gel, the following samples and controls were loaded:

- varying amounts(1000ng, 500ng, 250ng,100ng, 50ng, and 10ng) of target A(*aad1*) PCR product
- varying amounts(500ng, 1000ng, 250ng, 100ng, 50ng, and 10ng) of target B(*aad12*) PCR product,
- two dilutions(1ng and 0.25ng) of the positive control tfdA PCR product DNA
- 500ng of the negative control pBR322 DNA (PCR product~850bp)
- 10µg of the negative control salmon sperm gDNA X Bam H1 digested
- 10µg of the negative control salmon sperm gDNA X Eco R1 digested
- 10µg of the negative control salmon sperm gDNA X Hind III

Invitrogen 1Kb+ DNA ladder was used for sizing

Standard S. blot transfer procedures for the turbo blotter(descending transfer) were employed.

The nylon filter was UV linked and prehybridized (10mM EDTA, 6 X SSC, 0.5% SDS, 10% formamide, 5 X Denhardt's solution, and 100ug/ml denatured sheared salmon sperm DNA) for 4 hours at 42 degrees C. with shaking.

The filter was hybridized using 20 ml of the same buffer used for the prehybridization solution and 1000ng of T4 PNK 5' end labeled 32P tfdA DNA (total of 8,000,000dpm per 20ml) at 42 degrees C. for 24 hours.

The filter was rinsed at room temperature in 3 X SSC + 0.5% SDS for 5 minutes, followed by 2 rinses at room temperature (15 minutes each) in 3 X SSC + 0.1% SDS.

The **first wash** (3 changes) was carried out for **60 minutes** using **3 x SSC+ 0.1% SDS, at 35° C.**
The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1a)

The **second wash** (3 changes) was carried out for **2 hours** using **2 X SSC + 0.1% SDS at 68° C.**
The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1b)

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The **third wash** (3 changes) was carried out for **40 minutes** using **0.1 X SSC + 0.1% SDS at 68° C**. The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film.(D1c).

The autoradiograph obtained after **first wash (low stringency; D1a))** showed negative control background with very strong positive control and target sample signal. The autoradiograph obtained after the **second wash (moderate stringency; D1b)** demonstrates that this moderate stringency wash removed nearly all of the negative control background and left strong positive control and target sample signal. The autoradiograph obtained after the **third wash** (high stringency; D1c) demonstrates that the third wash totally removed the negative control signal and left very strong positive control signal and good target sample signal even down to 10ng of both target DNAs.

"Target A (aad1)" corresponds to a nucleic acid having the nucleotide sequence of SEQ ID No. 3.

"TfdA" corresponds to a nucleic acid having the nucleotide sequence of tfdA gene of Ralstonia eutropha (Streber et al. 1987; D2)

The application at page 3, paragraph 11 admits that "*TfdA catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction*". D3 (Fukumori and Hausinger) confirms the same.

The proteins encoded by TfdA gene and by the aad1 gene have 32% amino acid sequence identity (D4).

Even under stringent hybridization conditions, the nucleotide sequence of SEQ ID No. 3 specifically hybridizes to a nucleic acid encoding a protein having 2,4 degrading activity, having α-ketoglutarate-dependent dioxygenase reaction, and which was known from the art.

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4. Added Subject Matter (Articles 76(1), 123(2) EPC and 100 (c))

The subject matter of the granted claims goes beyond the disclosure of the application as filed. The claims thus contravene the requirements of Article 123(2) EPC. It should be noted that the current divisional application as filed appears to be an exact copy of the parental application as filed. At that time no claims were filed, but the originally filed claims were copied as items in the description on pages 159 to 168. Only in reply to the Invitation to remedy deficiencies of January 28, 2011, a set of claims to be pursued in the current application was filed. Accordingly, any subject matter identified as not being present in the divisional application will equally be subject matter extending beyond the scope of the originally filed application (Art 76(1) EPC and vice versa.

4.1 *Claim 1 contains subject matter not disclosed in the parent application as filed*

In his letter of January 28, 2011, the Patentee indicates originally filed claim 47 as basis for claim 1.

> Claim 1 as filed on January 28, 2011 and granted in unamended form, reads:
>
> *An isolated polynucleotide that encodes a protein* ***having alpha-ketoglutarate-dependent dioxygenase activity****, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:3, and SEQ ID NO:4, wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell (emphasis added).*

Originally filed claim 47 on the other hand read:

> *An isolated polynucleotide that encodes a protein* ***that enzymatically degrades a herbicide selecetd from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate****, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:3, and SEQ ID NO:4, wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell (emphasis added).*

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Patentee did not provide any basis for exchanging the teleological functional definition "that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate" for the biochemical functional definition " having alpha-ketoglutarate-dependent dioxygenase activity". There is no longer any requirement that the nucleic acid encodes a protein that degrades a particular class of herbicides, all that would now be required is that the encoded protein possesses alpha-ketoglutarate-dependent dioxygenase activity.

Admittedly, there is reference to proteins having alpha-ketogluturate-dependent dioxygenase activity, but always in the context of proteins that enzymatically degrade a herbicide selected from phenoxy auxins or aryloxyphenoxypropionate.

Interestingly, as is indicated in Figure 1 of the contested patent, the alpha-ketoglutarate is merely a co-substrate accepting the second oxygen atom from the O2-molecule, while the first oxygen atom is incorporated into the "real" substrate. Alpha-ketoglutarate as a co-substrate is common for many dioxygenases other than herbicide-degrading dioxygenases.

For instance, Hulse et al. 1978 (D5) describe an alpha-ketoglutarate dependent taurine dioxygenase involved in carnitine biosynthesis, while Eichorn et al. 1997 (D6) describe an alpha-ketoglutarate dependent taurine dioxygenase from *E. coli*.

Given the wide range of nucleic acids that can hybridize even under stringent conditions to the mentioned nucleotide sequences (see above), it will be clear that subject matter has been included in claim 1 which was not included in the application as originally filed at least by virtue of the absence of a reference to the substrate of the enzymes encoded by the claimed enzymes, and thus contravenes the requirements of Art 76(1).

4.2 ***Claims 2-10 contain subject matter not disclosed in the parent application as filed***

Other claims are dependent upon or refer to the subject matter of claim 1 and therefore also contravene the requirements of Art 76(1). Patentee referred to originally filed claims 45 and 46 as basis for granted claims 2 and 4; however, these originally filed claims were dependent upon originally filed claim 44 which required the protein to have "aroxyalkanoate dioxygenase activity", a

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feature which no longer is present in claim 1, thus leading to new combinations of features, extending beyond the content of the parent application as filed.

4.3 ***Claims 1 -10 contain subject matter not disclosed in the divisional application as filed.***

As indicated above, any subject matter not present in the parent application as filed is necessarily also not present in the divisional application as filed, and the deficiencies identified above under Art 76(1) are also deficiencies under Art 123(2).

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5 ***Priority entitlement (Article 87 EPC)***

As will be demonstrated below, the claims as granted are not entitled to the benefit of the priority date of 30 April 2004 (filing date of US60/567,052 D7). Consequently, the relevant date for determining the prior art base is the international filing date, i.e. May 2, 2005.

Comparing the application as filed to the priority document, it will be immediately apparent that the priority application contains less teaching than the parent and divisional application as filed. In particular, no claims as originally filed in the parent application (and included as items in the divisional application) are included in the priority document, as well as a number of Sequences, Figures and Examples are missing from the priority document.

Decision of the Enlarged Board of Appeal G2/98 has confirmed that an invention is only entitled to priority when the priority application clearly and unambiguously discloses that invention.

As set out above in connection with Art 100 (c), Art 76 and Art 123(2), the terms of the claims as granted cannot even be derived clearly and unambiguously from the parent or divisional application.

As the priority application contains even less teaching the parent application or divisional application as filed, the subject matter of the claims can also not be derived clearly and unambiguously from the priority application as filed. The subject matter of these claims is therefore not entitled to any of the claimed priorities in accordance with G2/98.

However, there are additional reasons why the claims are not entitled the benefit of the earlier priority dates.

As mentioned above, there are no claims included in the priority application. While some of the terms used in the current claims indeed can be found at various places in the application, there is no specific, clear and unambiguous disclosure of the combination of such features.

Claim 1 e.g. now claims isolated polynucleotides which

a) encode a protein having alpha-ketoglutate dependent dioxygenase activity
b) hybridize under stringent conditions with SEQ ID No. 3-5
c) are operably linked to a promoter that is functional in a plant cell

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The priority application only discloses proteins having alpha-ketoglutarate dependent dioxygenase activity in connection with degradation of phenoxy auxins or aryloxyphenoxyalkanoates. The priority application discusses different stringency conditions for hybridization (paragraphs 98-105), but does not make a specific link with using the sequences of SEQ ID No. 3-5. Furthermore, the priority application discusses different plant promoters in paragraphs 140 and 141 but not in connection with being operably linked specifically to nucleic acids hybridizing specifically with SEQ ID No. 3-5.

All other claims are dependent or refer to claim 1, and thus suffer from the same deficiency.

In addition, Claim 2 now claims that the polynucleotide comprises codon bias that is optimized for expression in a plant. Patentee has indicated that claim 2 finds basis in paragraphs 111 and Example 5/ paragraph 222. The corresponding passages in the priority application indicate a codon bias "more closely aligned to the plant target sequence" (paragraph 122).

Claim 3 now further specifies the encoded proteins have R-specific alpha-ketoglutarate dependent dioxygenase activity relative to a 2,4 D-herbicide. However, according to the application 2,4 D is an achiral phenoxy auxin (paragraph 74, page 10). No disclosure is provided for R-specific dioxygenases relative to 2,4-D.

Claim 4 refers to a specific nucleotide of SEQ ID No. 5. A sequence named SEQ ID No. 5 can be found in both the priority application and the application as filed, and even is designated in both cases as AAD-1(v3). However, whereas both sequences start with "ccatgg", the 3' ends seems to be different. SEQ ID No. 5 of the priority document is therefore not SEQ ID No. 5 as currently claimed.

Claim 6 is directed to a method for selecting a plant cell using a polynucleotide of claim 1 and growing in the presence of a phenoxy auxin or aryloxyphenoxyalkanoate herbicide. No such general language can be found in the priority application. Granted, there is a section on page 56 of the priority application entitled AAD-1(v3) as selectable marker, describing the ability to use the specific AAD-1 (v3) nucleotide sequence in connection with 2,4D as the selection agent. However, that is much more limited than claim 6.

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In conclusion, none of the subject matter of the claims can be derived clearly and unambiguously from the disclosure of the priority application.

The relevant date to establish the prior art base for all claims is therefore the international filing date of May 2, 2005. Consequently, documents D12 and D15 are prior art within the meaning of Art 54(2) EPC.

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6 Lack of Novelty (Articles 54 EPC and 100(a))

The subject-matter of several of the granted claims lacks novelty over the documents which will be discussed in detail in the following sections.

6.1 *Claims 1, 5, 7, 8 and 9 lack novelty over D8*

D8 is a first document describing transgenic plants expressing a bacterial detoxifying enzyme which plants are resistant to 2,4-D herbicide. D8 also describes isolated polynucleotides based on a tfdA gene from the soil bacterium *Alcaligenes eutrophus*, now renamed as *Ralstonia eutropha*, JM134 under control of a CaMV 35S promoter (see page 811, right column, section entitled "Construction of plasmids for constitutive and light induced DPAM expression in plants").

6.1.1 *Claim 1 lacks novelty over D8.*

As indicated above (section 3.1.1.) the *tfda* coding region (D2 ; reference 9 in D8) hybridizes under stringent conditions with a nucleic acid having the nucleotide sequence of SEQ ID No. 3 (and thus necessarily also with the complement). *tfdA* has α-ketoglutarate-dependent dioxygenase activity (application as filed page 3, paragraph 11). D8 discloses that the tfda region is operably linked to a CaMV35S promoter, which is a promoter functional in plant cells.

D8 thus anticipates claim 1 by disclosing all features of the subject matter of claim 1.

6.1.2 *Claim 5 lacks novelty over D8.*

Claim 5 further specifies the subject matter of claim 1 by requiring that the promoter is a promoter of viral origin. CaMV 35S is a promoter from cauliflower mosaic virus.

D8 thus anticipates claim 5 by disclosing all features of the subject matter of claim 5.

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6.1.3 *Claim 7 lacks novelty over D8.*

Claim 7 claims a method of detecting whether a plant comprises a polynucleotide of claim 1 comprising the steps of sampling the plant and assaying the sample for the presence of that polynucleotide.

As established in 6.1.1, D8 disclosed a polynucleotide sequence within the limits of claim 1. D8 further discloses assaying (leaf segments of) plants for the presence of that nucleotide sequence by Northern blot (page 813, left column, section "Transformation of tobacco with DPAM genes and transcript analysis") and by Southern blot (page 814, left column, first paragraph).

Claim 7 is thus anticipated by D8.

6.1.4 *Claim 8 lacks novelty over D8.*

Claim 8 further defines the method of claim 7 by specifying that the method comprises assaying a sample from a plant comprising the polynucleotide of claim 1 for the presence of a protein encoded by the polynucleotide.

Claim 8 does not specify how the presence of the protein has to be determined; ergo it can be determined by any means including the phenotypic effect of the presence of such protein.

D8 discloses a test using leaf discs from transgenic plants to test for their ability to regenerate shoots on media containing elevated levels of 2,4-D (page 813, left column, third paragraph.

D8 therefore discloses a method as claimed in claim 8.

6.1.5 *Claim 9 lacks novelty over D8.*

Claim 9 further specifies the method of claim 7 by defining that a PCR primer or probe is used to detect the polynucleotide. Both Northern and Southern hybridization use labelled probes to detect the specific sequences.

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Claim 9 is anticipated by D8

6.2 *Claims 1 and 5 lack novelty over D9*

D9 is another document describing cloning of a tfdA gene under control of CaMV35S promoter and expressing it in plants.

6.2.1 *Claim 1 lacks novelty over D9.*

As indicated above (section 3.1.1.) the *tfda* coding region hybridizes under stringent conditions with a nucleic acid having the nucleotide sequence of SEQ ID No. 3. *tfdA* has α-ketoglutarate-dependent dioxygenase activity (application as filed page 3, paragraph 11). D8 discloses that the tfda region is operably linked to a CaMV35S promoter (page 535, left column, line 4), which is a promoter functional in plant cells.

D9 thus anticipates claim 1 by disclosing all features of the subject matter of claim 1.

D9 thus anticipates claim 1.

6.2.2 *Claim 5 lacks novelty over D9*

Claim 5 further specifies the subject matter of claim 1 by requiring that the promoter is a promoter of viral origin. CaMV 35S is a promoter from cauliflower mosaic virus.

D8 thus anticipates claim 5 by disclosing all features of the subject matter of claim 5.

6.3 *Claims 1, 5, 7 and 9 lack novelty over D10.*

D10 is yet another publication wherein the tfdA gene of *A. eutrophus* JMP34 is cloned under the control of the CaMV 35S promoter (page 163, left column, second paragraph, lines 1 to 4). Floral buds and young leaves of transgenic plants were subjected to either PCR analysis or Southern blot analysis (see page 164, last sentence of the second paragraph) and Figure 2A on page 165.

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D10 therefore also anticipates claims 1, 5, 7 and 9 as elaborated under 6.1.

6.4 *Claims 1, 5, 7 and 9 lack novelty over D11.*

D11 is also a publication wherein the tfdA gene of *A. eutrophus* is cloned under the control of the CaMV 35S promoter (page 645 right column, last paragraph; page 646, left column, lines 14 to 16). Young leaves of transgenic plants were subjected to PCR analysis (see page 646, right column, section " Molecular analysis of plant DNA".

D11 therefore also anticipates claims 1, 5, 7 and 9 as elaborated under 6.1.

6.5 *Claims 1, 5, 7 and 9 lack novelty over D12.*

D12 is the last publication cited wherein the tfdA gene of *A. eutrophus* is cloned under the control of the CaMV 35S promoter (page 367 right column, third paragraph). Leaves from transgenic plants were subjected to Southern blot analysis (see page 367, right column, last two lines and Figure 1 on page 368).

D12 therefore also anticipates claims 1, 5, 7 and 9 as elaborated under 6.1.

6.6 *Conclusion*

Claims 1, 5,7 and 9 are anticipated by one or more of documents D8, D9, D10, D11 or D12.

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7 Inventive step (Articles 56 EPC and 100(b))

7.1 Claims which are strictly speaking considered novel over any one of D8 to D12 lack an inventive step.

Claims 1, 5, 7, 8 and 9 are anticipated by one or more of documents D8 to D12. For remaining claims 2, 3, 4 and 10 Opponent submits they lack an inventive step over the prior art.

7.1.1 *Claim 2 lacks inventive step over D8 (or any of D9, D10, D11, D12) in combination with D18.*

Claim 2 merely specifies that the polynucleotide of claim 1 comprises a codon bias that is optimized for expression in a plant.

Such a codon usage optimization (although apparently not strictly required for activity) was quite common in the art at the filing date of the application. The application itself refers to D18 for details of the method of codon usage optimization (page 58, paragraph 224) and the codon optimization of AAD1 (v3) was outsourced to an external vendor (Picoscript; see page 60, paragraph 229). The subject matter of claim 2 therefore amounts to nothing more than a workshop adaptation of the teaching of the prior art.

7.1.2 *Claim 3 lacks an inventive step over D8 (or any of D9, D10, D11, D12) in combination with D15.*

D8 does not disclose that polynucleotide can encode a protein have stereospecific (R-specific) activity towards a phenoxy auxin (Note that claim 3 actually requires R-specific cleavage of 2,4 D-herbicide, which appears to be a contradiction.)

The technical problem therefore can be formulated as providing a polynucleotide under control of a plant-expressible promoter encoding a protein with stereospecific (R-specific) activity towards a phenoxy auxin.

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D15 discloses such coding regions. In fact, D15 discloses a coding region that is identical to SEQ ID No. 3 (except for an ambiguous Y nucleotide instead of C, see D13 and D14) and the encoded protein is identical.

A person skilled in the art would have substituted the tfdA gene in the polynucleotide of e.g. D8 for the coding region encoding stereospecific protein as in D15 to arrive at the claimed subject matter without inventive contribution.

7.1.3 *Claim 4 lacks an inventive step over D8 (or any of D9, D10, D11, D12) in combination with D14.*

D8 discloses a polynucleotide with a tfdA coding region under control of a promoter functional in plant cells, rather than the rdpA of SEQ ID No. 5.

The technical problem can thus be formulated as providing alternative phenoxy auxin herbicide degrading enzyme encoding polynucleotide constructs.

As elaborated above, D14 discloses such a nucleotide sequence, which has been codon optimized according to general knowledge to arrive at the nucleotide sequence of SEQ ID No. 5.

No inventive skill is needed to arrive at the subject matter of claim 4.

7.1.4 *Claim 10 lacks an inventive step over D8 (or any of D9, D10, D11, D12) in combination with common general knowledge.*

Claim 10 depends on claim 8 and further specifies that method as detecting the presence of the encoded protein by using an antibody.

Even the specification acknowledges that the preparation of antibodies to the proteins disclosed therein can be "readily prepared using standard procedures" (page 26, paragraph 113). These further features can therefore not bestow an inventive activity on the claimed subject matter.

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8 Sufficiency of Disclosure (Articles 83 EPC and 100(b))

The invention as claimed in the patent as granted is insufficiently disclosed and therefore contravenes the requirements of Article 83 EPC.

In the European Search opinion of June 29, 2011, then pending claim 10 was objected under Art 83 EPC. According to the EPO, *"[t]he claims cover all methods having [..][the] characteristic or property [of R-specific alpha-ketoglutarate-dependent dioxygenase], whereas the application provides support in the meaning of Art 84 EPC for only a very limited number of such methods, i.e. those employing a polynucleotide having SEQ ID NO 3-5 which encode a polypeptide having SEQ ID NO: 9. Hence, the subject matter of claim 10 lacks technical support in the sense of Art 84 EPC, and the application lacks disclosure as required in Art 83 EPC".*

In the reply of January 27, 2012, Patentee introduced current claim 3, dependent on claim 1 and argued that *"the R-specific alpha-ketoglutarate-dependent dioxygenase activity is limited to those scenarios involving employing a polynucleotide having SEQ NO 3-5".* While strictly speaking it is correct that the now claimed isolated nucleotide sequences are identified by employing hybridization with any of SEQ ID No. 3-5, this does not remedy the issue identified by the EPO that the application provides support and disclosure only for a very limited number of methods and polynucleotides i.e. those having SEQ ID No. 3-5 which encode a polypeptide having SEQ ID No. 9.

As demonstrated above, the definition of the claimed polynucleotides by virtue of capacity to hybridize under stringent conditions, still encompasses a large genus of polynucleotides including polynucleotides encoding a protein having as little as 32% amino acid sequence identity, and is thus not congruent with the limited disclosure by the application.

Therefore, at least claim 3 contravenes the requirements of Article 83 EPC. However, a similar objection can be raised mutatis mutandis against claim 1 or claim 6.

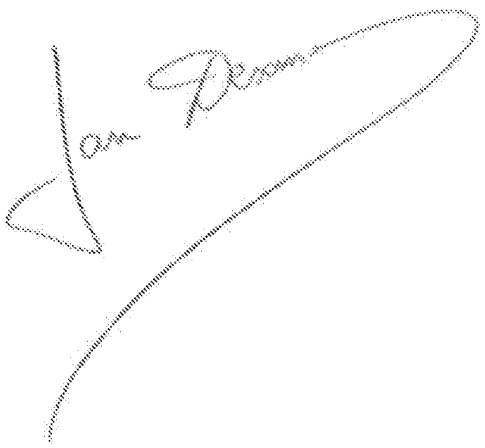
Additionally, Claim 3 specifies the encoded proteins as having R-specific alpha-ketoglutarate dependent dioxygenase activity relative to a 2,4 D-herbicide. However, according to the application

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2,4 D is an achiral phenoxy auxin (paragraph 74, page 10). No disclosure is provided for R-specific dioxygenases relative to 2,4-D.

9 Conclusion

The subject matter of the claims as granted does not meet the requirements of the EPC. The patent should be revoked in its entirety.



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Opposition by

Bayer CropScience N.V.

against

European Patent EP-B-2 308 977

granted in the name of Dow AgroSciences LLC.

NOTICE OF OPPOSITION

1. Introduction and Requests

Opposition against European Patent n° EP 2 308 977, granted in the name of Dow AgroSciences LLC, entitled "NOVEL HERBICIDE RESISTANCE GENE", is hereby filed by Bayer CropScience N.V., J.E. Mommaertslaan 14, BE-1831 Diegem, Belgium.

It is requested that the patent as granted is revoked under Articles 100(a), 100(b) and Art 100(c) EPC.

Oral proceedings are also requested under Article 116 EPC in the event that the Opposition Division does not intend to revoke the patent on the basis of the written submissions.

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2. Cited Documents

In support of the opposition, Opponent cites the following documents:

D1: Autoradiographs from hybridization experiments between AAD-1 and tfdA nucleic acids

D2: Streber, Timmis and Zenk, 1987, J. Bacteriology 169(7): 2950-2955

D3: Fukumori and Hausinger 1993 J. Bacteriology 175(7): 2083-2086

D4: amino acid sequence alignment between TFDA and AAD1 protein

D5: Hulse, Ellis and Henderson 1978 J. Biol. Chemistry 253: 1654-1659

D6: Eichorn, van der Ploeg, Kertesz and Leisinger 1997 J. Biol. Chemistry 272 (37): 23031-23036

D7: priority document USSN 60/567,052

D8: Streber and Willmitzer 1989 Biotechnology 7: 811-816

D9: Lyon, Llewellyn, Huppatz, Dennis and Peacock 1989 Plant Molecular Biology 13 : 533-540

D10: Lyon, Cousins, Llewellyn and Dennis 1993 Transgenic Research 2 : 162-169

D11: Bayley, Trolinder, Ray, Morgan, Quisenberry and Ow 1992 Theor. Appl. Genet 83:645-649

D12 : Bisht, Burma and Pental 2004 Current Science 87(3) : 367-370

D13 : alignment of the nucleotide sequence of SEQ ID No. 3 of the contested patent with the nucleotide sequence of entry AF516751 (Rhodoferax sp P230 rdpA gene).

D14: AF516751 nucleotide sequence, version of Jun 26, 2003

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D15: Schleinitz, Kleinsteuber, Vallaeys and Babel 2004 Applied and Environmental Microbiology 70(9): 5357-5365

D16: alignment of the nucleotide sequence of SEQ ID No. 3 of the contested patent with the nucleotide sequence of entry AJ628859 (Sphingomonas herbicidovorans tmpA gene)

D17: AJ628859 nucleotide sequence version of Mar 3 2004.

D18: WO 97/13402

D19: WO87/05629

D20: WO 03/013224

D21: US 5,463,175

D22: US2003/0135879

D23: WO96/33270

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3. Interpretation of the claims as granted

3.1 *Terms of the claims*

In the below analysis of the claims with the patentability requirements, the following term in the claims has been construed in accordance with the teaching of the application, or in absence thereof, been given its broadest meaning in accordance with established case-law (T686/96; T79/96).

3.1.1 "hybridizing under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID No. 9 ".

The application as filed, page 30, paragraph 127, provides the following guidance: "*As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants*".

The application further refers to standard methods as e.g. defined by Maniatis et al. 1982 to perform hybridization of immobilized DNA on Southern blots with 32P-labeled gene-specific probes. In paragraph 131, spanning pages 30 and 31, examples of salt conditions are provided to influence the stringency in hybridizations when using labeled DNA fragments longer than 70 bases in length, including low, moderate or high stringent conditions.

Similarly, paragraph 124 on page 29 provides guidance on hybridization. Specifically, it is indicated that hybridization can be followed by subsequent washes under conditions of low, moderate or high stringency, which are achieved by using first washes with 2xSSC/01% SDS for 15 minutes at room temperature (low); subsequently with 0.1%SSC/0.1%SDS at room temperature (moderate) and then with 0.1%SSC/0.1%SDS for 30 minutes at 55°C. The application also teaches that "higher stringency can be achieved by lowering the salt concentration and/or by raising the temperature".

It is important to realize that the claims merely require "hybridization" and "stringent" conditions, which is not to be equated with "specific hybridization" and "high stringent" conditions.

Document D1 consists of three autoradiographs, resulting from a hybridization experiment performed by an independent contracting lab.

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Using a 0.8% TBE agarose gel, the following samples and controls were loaded:

- varying amounts (1000ng, 500ng, 250ng, 100ng, 50ng, and 10ng) of target A (*aad1*) PCR product
- varying amounts (500ng, 1000ng, 250ng, 100ng, 50ng, and 10ng) of target B (*aad12*) PCR product,
- two dilutions (1ng and 0.25ng) of the positive control tfdA PCR product DNA
- 500ng of the negative control pBR322 DNA (PCR product~850bp)
- 10µg of the negative control salmon sperm gDNA X Bam H1 digested
- 10µg of the negative control salmon sperm gDNA X Eco R1 digested
- 10µg of the negative control salmon sperm gDNA X Hind III

Invitrogen 1Kb+ DNA ladder was used for sizing

Standard S. blot transfer procedures for the turbo blotter (descending transfer) were employed.

The nylon filter was UV linked and prehybridized (10mM EDTA, 6 X SSC, 0.5% SDS, 10% formamide, 5 X Denhardt's solution, and 100ug/ml denatured sheared salmon sperm DNA) for 4 hours at 42 degrees C. with shaking.

The filter was hybridized using 20 ml of the same buffer used for the prehybridization solution and 1000ng of T4 PNK 5' end labeled 32P tfdA DNA (total of 8,000,000dpm per 20ml) at 42 degrees C. for 24 hours.

The filter was rinsed at room temperature in 3 X SSC + 0.5% SDS for 5 minutes, followed by 2 rinses at room temperature (15 minutes each) in 3 X SSC + 0.1% SDS.

The **first wash** (3 changes) was carried out for **60 minutes** using **3 x SSC+ 0.1% SDS, at 35° C.**
The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1a)

The **second wash** (3 changes) was carried out for **2 hours** using **2 X SSC + 0.1% SDS at 68° C.**
The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1b)

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The **third wash** (3 changes) was carried out for **40 minutes** using **0.1 X SSC + 0.1% SDS at 68° C**. The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1c).

The autoradiograph obtained after **first wash (low stringency; D1a)** showed negative control background with very strong positive control and target sample signal. The autoradiograph obtained after the **second wash (moderate stringency; D1b)** demonstrates that this moderate stringency wash removed nearly all of the negative control background and left strong positive control and target sample signal. The autoradiograph obtained after the **third wash** (high stringency; D1c) demonstrates that the third wash totally removed the negative control signal and left very strong positive control signal and good target sample signal even down to 10ng of both target DNAs.

"Target A (aad1)" corresponds to a nucleic acid encoding a protein of SEQ ID No. 9

"TfdA" corresponds to a nucleic acid having the nucleotide sequence of tfdA gene of Ralstonia eutropha (Streber et al. 1987; D2)

The application at page 3, paragraph 11 admits that "*TfdA catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction*". D3 (Fukumori and Hausinger) confirms the same.

The proteins encoded by TfdA gene and by the aad1 gene have 32% amino acid sequence identity (D4).

Even under stringent hybridization conditions, the nucleotide sequence of SEQ ID No. 3 specifically hybridizes to a nucleic acid encoding a protein having 2,4 degrading activity, and which was known from the art.

3.1.2 "a method of controlling at least one weed in an area".

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There appears to be no specific conditions which apply to term "controlling weeds" or "area". Accordingly, it can be interpreted as applying a herbicide to a plant, including in greenhouse or experimental conditions.

4. Added Subject Matter (Articles 76(1), 123(2) EPC and 100 (c))

The subject matter of the granted claims goes beyond the disclosure of the application as filed. The claims thus contravene the requirements of Article 123(2) EPC. It should be noted that the current divisional application as filed appears to be an exact copy of the parental application as filed. At that time no claims were filed, but the originally filed claims were copied as items in the description. Only on January 27, 2011, a set of claims to be pursued in the current application was filed. Accordingly, any subject matter identified as not being present in the divisional application will equally be subject matter extending beyond the scope of the originally filed application (Art 76(1) EPC and vice versa.

4.1 *Claims 1 and 19 contains subject matter not disclosed in the parent application as filed*

In his letter of January 27, 2011, the Patentee indicates originally filed claim 13 as basis for claim 1, together with paragraphs 112, 118 and 124-131 as support for hybridization conditions and paragraphs 5 and 17 as support for "area".

Claim 1 as filed on January 27, 2011 and granted in unamended form, reads:

> *A method of controlling at least one weed in an area, wherein said area comprises at least one transgenic plant comprising an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID No:9, where said plant expresses said polynucleotide and produces said protein, wherein said method comprises applying said herbicide to said area.*

Originally filed claim 13 on the other hand reads:

> *A method of controlling at least one weed in* ***a field****, wherein said field contains at least one plant of claim 5, wherein said method comprises applying to at least one portion of said field*

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a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide (emphasis added).

Originally filed claim 5 reads:

A transgenic plant comprising a plurality of cells according to claim 1, wherein expression of said polynucleotide renders said cells tolerant to an aryloxyalkanoate herbicide.

Originally filed claim 1 reads:

A transgenic plant cell comprising ***a polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO: 9 or a variant of SEQ ID NO:9 wherein said variant has aryloxyalkanoate dioxygenase activity, at least one amino acid deletion or conserved substitution , and at least 95% sequence identity with SEQ ID NO:9*** *(emphasis added).*

In other words, the method of claim 13 as originally filed was limited to methods for controlling weeds in a field using plants expressing AAD-1 or a highly related variant thereof.

Paragraphs 112, 118 and 124-131 describe hybridization conditions and indicate that the class of proteins can be defined by hybridization properties of the encoding polynucleotide, but does not make reference to a method of controlling weeds in a field of an area.

Given the wide range of nucleic acids that can hybridize even under stringent conditions to the mentioned nucleotide sequence (see above), it will be clear that subject matter has been included in claim 1 which was not included in the application as originally filed and thus contravenes the requirements of Art 76(1).

It is telling that Patentee provided as support for claim 2 paragraphs 20, 78 and 89. However each of these citations are explicitly linked to plants comprising an AAD-1 gene.

- *The subject invention also includes methods of controlling weeds wherein said methods comprising applying one or more AOPP, phenoxy auxin, or other aryloxyalkanoate herbicides to plants comprising an AAD-1 gene (paragraph 28)*
- *Plants containing AAD-1 alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides (paragraph 78)*

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- *The subject invention also includes methods of controlling weeds wherein said methods comprise applying an AOPP herbicide and/or phenoxy auxin herbicide to plants comprising an AAD-1 gene (paragraph 89).*

The term "area" is used in paragraphs 5 and 7 only in connection with glyphosate use and occurrence of glyphosate tolerant weeds and provides no basis for the substitution in claim 1 of the word "field".

The new combination of features in claim 1 cannot be unambiguously and directly derived from the application as originally filed.

The argumentation applies mutatis mutandis to the subject matter of claim 19.

4.2 ***Claims 2-18 and 20 contain subject matter not disclosed in the parent application as filed***

Claims 2-18 are dependent upon or refer to the subject matter of claim 1; claim 20 is dependent upon claim 19. These claims therefore also contravene the requirements of Art 76(1).

4.3 ***Claims 1 -20 contain subject matter not disclosed in the divisional application as filed.***

As indicated above, any subject matter not present in the parent application as filed is necessarily also not present in the divisional application as filed, and the deficiencies identified above under Art 76(1) are also deficiencies under Art 123(2).

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5 ***Priority entitlement (Article 87 EPC)***

As will be demonstrated below, the claims as granted are not entitled to the benefit of the priority date of 30 April 2004 (filing date of US60/567,052 D7). Consequently, the relevant date for determining the prior art base is the international filing date, i.e. May 2, 2005.

Comparing the application as filed to the priority document, it will be immediately apparent that the priority application contains less teaching than the parent and divisional application as filed. In particular, no claims as originally filed in the parent application (and included as items in the divisional application) are included in the priority document, as well as a number of Sequences, Figures and Examples are missing from the priority document.

Decision of the Enlarged Board of Appeal G2/98 has confirmed that an invention is only entitled to priority when the priority application clearly and unambiguously discloses that invention.

As set out above in connection with Art 100 (c), Art 76 and Art 123(2), the terms of the claims as granted cannot even be derived clearly and unambiguously from the parent or divisional application.

As the priority application contains even less teaching than the parent application or divisional application as filed, the subject matter of the claims can also not be derived clearly and unambiguously from the priority application as filed. The subject matter of these claims is therefore not entitled to any of the claimed priorities in accordance with G2/98.

However, there are additional reasons why the claims are not entitled the benefit of the earlier priority dates.

As mentioned above, there are no claims included in the priority application. While some of the terms used in the current claims indeed can be found at various places in the application, there is no specific, clear and unambiguous disclosure of the combination of such features.

The closest disclosure in the priority application of a method of controlling weeds is in the paragraphs corresponding to paragraphs 20, 78 and 89 of the originally filed application, which however, as mentioned above are specifically limited to plant comprising the AAD-1 gene.

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Claims 2-18 are dependent or refer to claim 1, and thus suffer from the same deficiency.

Additionally, Claim 19 refers to a specific nucleotide of SEQ ID No. 5. A sequence identified as SEQ ID No. 5 can be found in both the priority application and the application as filed, and even is designated in both cases as AAD-1(v3). However, whereas both sequences start with "ccatgg", the 3' ends seems to be different. SEQ ID No. 5 of the priority document is therefore not SEQ ID No. 5 as currently claimed.

Claim 20 is dependent upon claim 19.

In conclusion, none of the subject matter of the claims can be derived clearly and unambiguously from the disclosure of the priority application.

The relevant date to establish the prior art base for all claims is therefore the international filing date of May 2, 2005. Consequently, documents D12 and D15 are prior art within the meaning of Art 54(2) EPC.

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6 Lack of Novelty (Articles 54 EPC and 100(a))

The subject-matter of several of the granted claims lacks novelty over the documents which will be discussed in detail in the following sections.

6.1 *Claims 1 to 3, 11, 12 and 18 lack novelty over D8*

D8 is a first document describing transgenic plants expressing a bacterial detoxifying enzyme which plants are resistant to 2,4-D herbicide. D8 also describes isolated polynucleotides based on a tfdA gene from the soil bacterium *Alcaligenes eutrophus*, now renamed as *Ralstonia eutropha*, JM134 under control of a CaMV 35S promoter (see page 811, right column, section entitled "Construction of plasmids for constitutive and light induced DPAM expression in plants"). Transgenic plants were grown in the greenhouse and sprayed with 2,4-D (page 813, right column, first paragraph).

6.1.1 *Claim 1 lacks novelty over D8.*

As indicated above (section 3.1.1.) the *tfda* coding region (D2 ; reference 9 in D8) hybridizes under stringent conditions with a nucleic acid encoding a protein of SEQ ID No. 9 (and thus necessarily also with the complement). *tfdA* has 2,4 D degrading activity (application as filed page 3, paragraph 11). 2,4D herbicide has been sprayed on the transgenic plants, in the greenhouse.

D8 thus anticipates claim 1 by disclosing all features of the subject matter of claim 1.

6.1.2 *Claim 2 lacks novelty over D8.*

Claim 2 further specifies the subject matter of claim 1 by requiring that the herbicide is applied to the plant. D8 discloses spraying of transgenic plants with 2,4 D.

D8 thus anticipates claim 2 by disclosing all features of the subject matter of claim 5.

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6.1.3 *Claim 3 lacks novelty over D8.*

Claim 3 further specifies the subject matter of claim 1 by requiring that the herbicide is a phenoxy herbicide , preferably 2,4-D. D8 discloses spraying of transgenic plants with 2,4 D.

Claim 7 is thus anticipated by D8.

6.1.4 *Claim 11 lacks novelty over D8.*

Claim 11 further defines the method of claim 1 by specifying that herbicide is applied inter alia post-emergence. D8 discloses the spraying of transgenic plants, i.e. after the plants have emerged.

D8 therefore discloses a method as claimed in claim 11.

6.1.5 *Claim 12 lacks novelty over D8.*

Claim 12 further specifies the method of claim 1 by defining that a seed is allowed to be grown in the area to which the herbicide is to be applied.

D8 (page 814, left column first paragraph) discloses progeny seeds of the transgenic plants, germinating into plants, 20 of which were sprayed with 2,4-D.

Claim 12 is anticipated by D8

6.1.6 *Claim 18 lacks novelty over D8.*

Claim 18 further specifies the method of claim 1 by requiring that one or more herbicides are applied to the area.

D8 discloses applying one herbicide (2,4-D) the area.

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6.2 ***Claims 1 to 3, 11, 12 and 18 lack novelty over D9***

D9 is another document describing cloning of a tfdA gene under control of CaMV35S promoter and expressing it in plants. D9 discloses that the tfda region is operably linked to a CaMV35S promoter (page 535, left column, line 4), which is a promoter functional in plant cells. Progeny of transgenic plants were germinated and grown for 4 to 8 weeks in soil, prior to spraying with 2,4D herbicide (pages 538 left column, second section). D9 anticipates claims 1 to 3, 11, 12 and 18 for the same reasons as explained under 6.1.

6.3 ***Claims 1 to 3, 11, 12 and 18 lack novelty over D10***

D10 is yet another publication wherein the tfdA gene of *A. eutrophus* JMP34 is cloned under the control of the CaMV 35S promoter (page 163, left column, second paragraph, lines 1 to 4). Transgenic plants comprising this transgene are generated and sprayed with 2,4-D herbicide (page 167, left column, last paragraph; right column, end of the first paragraph). D10 thus anticipates claims 1 to 3, 11, 12 and 18 for the same reasons as explained under 6.1.

6.4 ***Claims 1 to 3, 11, 12 and 18 lack novelty over D11.***

D11 is a publication wherein the tfdA gene of *A. eutrophus* is cloned under the control of the CaMV 35S promoter (page 645 right column, last paragraph; page 646, left column, lines 14 to 16). Transgenic tobacco and cotton plants were generated and sprayed with 2,4-D herbicide (see page 648, Fig 2; page 648 right column paragraph preceding the concluding remarks).

D11 therefore also anticipates claims 1 to 3, 11, 12 and 18 as elaborated under 6.1.

6.5 ***Claims 1 to 3, 11, 12 and 18 lack novelty over D12.***

D12 is the last publication cited wherein the tfdA gene of *A. eutrophus* is cloned under the control of the CaMV 35S promoter (page 367 right column, third paragraph). Transgenic mustard plants were generated and sprayed in field trials with 2,4-D herbicide (page 368, right column, third paragraph and Table 2).

D12 therefore also anticipates claims 1 to 3, 11, 12 and 18 as elaborated under 6.1.

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6.6 *Conclusion*

Claims 1 to 3, 11, 12 and 18 are anticipated by one or more of documents D8, D9, D10, D11 or D12.

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7 Inventive step (Articles 56 EPC and 100(b))

7.1 Claims which are strictly speaking considered novel over any one of D8 to D12 lack an inventive step.

Claims 1 to 3, 11, 12 and 18 are anticipated by one or more of documents D8 to D12. For remaining claims 2, 3, 4 and 10 Opponent submits they lack an inventive step over the prior art.

7.1.1 *Claims 5 to 9 lack inventive step over D8 (or any of D9, D10, D11, D12) in combination with any one of D19, D20, D21 or D22 .*

Claims 5 to 9 further define the method of claim 1 by specifying that the plants may comprise other herbicide tolerance genes, particularly glufosinate tolerance genes, glyphosate tolerance genes, dicamba tolerance genes and genes conferring tolerance to ALS inhibitor herbicides and that the corresponding herbicides may also be used.

Documents D8 to D12 do not disclose such additional herbicide genes. However, the application does not claim any interaction between the presence of the phenoxy auxin degrading transgene and the other herbicide tolerance genes. The problems can thus be separated and merely amounts to the provision of plants with these other tolerance genes.

It will be clear that these other tolerance genes, and their application for generating herbicide tolerant plants to be used in weed control programs is well know from the prior art, including D19 (glufosinate tolerance) D20 (glufosinate tolerance) D21 (glyphosate tolerance) D22 (dicamba tolerance) and D23 (ALS inhibitor tolerance).

7.1.2 *Claim 13 lacks an inventive step over D8 (or any of D9, D10, D11, D12 .*

Claim 13 merely defines that the method of claim 1 is applied to maize plants. No specific advantage is alleged for applying the method to corn plants, rather than to cotton, mustard, or tobacco plants and the choice of a particular plant cannot provide an inventive step.

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7.1.3 *Claim 14 lacks an inventive step over D8 (or any of D9, D10, D11, D12) in combination with D14.*

D8 discloses a method of weed control using transgenic plants polynucleotide with a tfdA coding transgene, rather than the rdpA of SEQ ID No. 9, disclosed in the contested patent.

The technical problem can thus be formulated as providing a weed control method using alternative phenoxy auxin herbicide degrading enzyme encoding polynucleotide constructs and transgenic plants.

D15 discloses such coding regions involved in phenoxy auxin herbicide degradation. In fact, D15 discloses a coding region that is identical to SEQ ID No. 3 (except for an ambiguous Y nucleotide instead of C, see D13 and D14) and the encoded protein (SEQ ID No. 9) is identical.

A person skilled in the art would have substituted the tfdA gene in the polynucleotide of e.g. D8 for the coding region encoding stereospecific protein as in D15 to arrive at the claimed subject matter with reasonable expectation of success in view of the successes reported in any of D8 to D12.

No inventive skill is needed to arrive at the subject matter of claim 14.

8 Sufficiency of Disclosure (Articles 83 EPC and 100(b))

The invention as claimed in the patent as granted is insufficiently disclosed and therefore contravenes the requirements of Article 83 EPC.

the application provides support and disclosure only for a very limited number of methods and polynucleotides i.e. those having SEQ ID No. 3-5 which encode a polypeptide having SEQ ID No. 9.

As demonstrated above, the definition of the claimed polynucleotides by virtue of capacity to hybridize under stringent conditions, still encompasses a large genus of polynucleotides including polynucleotides encoding a protein having as little as 32% amino acid sequence identity, and is thus not congruent with the limited disclosure by the application.

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polynucleotides encoding a protein having as little as 32% amino acid sequence identity, and is thus not congruent with the limited disclosure by the application.

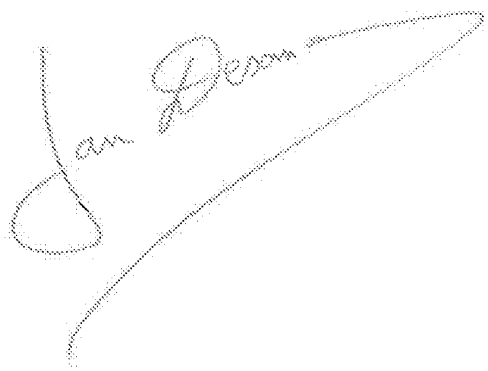
Additionally, Claim 17 requires the application of a "parent herbicide". The term parent herbicide is used in the application only in connection with the metabolization of 2,4D herbicide through the enzymatic action of AAD-1. It is unclear, nor does the application provide any teaching on how the term "parent herbicide" connects with the method of controlling weeds in an area. Claim 17 is thus not sufficiently disclosed.

Remark

Opponent further remarks that claim 14 of the current patent claims the same subject matter as claim 16 of EP 1 740 039 granted on the parent application. It is an accepted principle in most patent systems that two patents cannot be granted to the same applicant for one invention. The Enlarged Board of Appeal has accepted obiter dictum that the principle of the prohibition on double patenting is based on the notion that an applicant has no legitimate interest in proceedings leading to the grant of a second patent for the same subject-matter if he already possesses one granted patent for that subject-matter (see G 1/05, and G 1/06).

Conclusion

The subject matter of the claims as granted does not meet the requirements of the EPC. The patent should be revoked in its entirety.



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Opposition by

Bayer CropScience NV

against

European Patent **EP-B-2 319 932**
granted in the name of Dow AgroSciences LLC.

NOTICE OF OPPOSITION

1. Introduction and Requests

Opposition against European Patent n° EP 2 319 932, granted in the name of Dow AgroSciences LLC, entitled "NOVEL HERBICIDE RESISTANCE GENE", is hereby filed by Bayer CropScience NV, J.E. Mommaertslaan 14, BE-1831 Diegem, Belgium.

It is requested that the patent as granted is revoked under Articles 100(a), 100(b) and Art 100(c) EPC.

Oral proceedings are also requested under Article 116 EPC in the event that the Opposition Division does not intend to revoke the patent on the basis of the written submissions.

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2. Cited Documents

In support of the opposition, Opponent cites the following documents:

D1: Autoradiographs from hybridization experiments between AAD-1 and tfdA nucleic acids

D2: Streber, Timmis and Zenk, 1987, J. Bacteriology 169(7): 2950-2955

D3: Fukumori and Hausinger 1993 J. Bacteriology 175(7): 2083-2086

D4: amino acid sequence alignment between TFDA and AAD1 protein

D5: Hulse, Ellis and Henderson 1978 J. Biol. Chemistry 253: 1654-1659

D6: Eichorn, van der Ploeg, Kertesz and Leisinger 1997 J. Biol. Chemistry 272 (37): 23031-23036

D7: priority document USSN 60/567,052

D8: Streber and Willmitzer 1989 Biotechnology 7: 811-816

D9: Lyon, Llewellyn, Huppatz, Dennis and Peacock 1989 Plant Molecular Biology 13: 533-540

D10: Lyon, Cousins, Llewellyn and Dennis 1993 Transgenic Research 2: 162-169

D11: Bayley, Trolinder, Ray, Morgan, Quisenberry and Ow 1992 Theor. Appl. Genet 83: 645-649

D12 : Bisht, Burma and Pental 2004 Current Science 87(3): 367-370

D13 : alignment of the nucleotide sequence of SEQ ID No. 3 of the contested patent with the nucleotide sequence of entry AF516751 (Rhodoferax sp P230 rdpA gene).

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D14: AF516751 nucleotide sequence, version of Jun 26, 2003

D15: Schleinitz, Kleinsteuber, Vallaeys and Babel 2004 Applied and Environmental Microbiology 70(9): 5357-5363

D16: alignment of the nucleotide sequence of SEQ ID No. 3 of the contested patent with the nucleotide sequence of entry AJ628859 (Sphingomonas herbicidovorans tapA gene)

D17: AJ628859 nucleotide sequence version of Mar 3 2004

D18: WO 97/13402

D19: WO87/05629

D20: WO 03/013224

D21: US 5,463,175

D22: US2003/0135879

D23: WO96/33270

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3. Interpretation of the claims as granted

3.1 *Terms of the claims*

In the below analysis of the claims with the patentability requirements, the following term in the claims has been construed in accordance with the teaching of the application, or in absence thereof, been given its broadest meaning in accordance with established case-law (T686/96; T79/96).

3.1.1 "hybridizing under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID No. 9 ".

The application as filed, page 30, paragraph 127, provides the following guidance: "*As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants*".

The application further refers to standard methods as e.g. defined by Maniatis et al. 1982 to perform hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes. In paragraph 131, spanning pages 30 and 31, examples of salt conditions are provided to influence the stringency in hybridizations when using labeled DNA fragments longer than 70 bases in length, including low, moderate or high stringent conditions.

Paragraph 124 on page 29 provides guidance on hybridization. Specifically, it is indicated that hybridization can be followed by subsequent washes under conditions of low, moderate or high stringency, which are achieved by using first washes with 2xSSC/01% SDS for 15 minutes at room temperature (low); subsequently with 0.1%SSC/0.1%SDS at room temperature (moderate) and then with 0.1%SSC/0.1%SDS for 30 minutes at 55°C. The application also teaches that "higher stringency can be achieved by lowering the salt concentration and/or by raising the temperature".

It is important to realize that the claims merely require "hybridization" and "stringent" conditions, which is not to be equated with "specific hybridization" and "high stringent" conditions.

Document D1 consists of three autoradiographs, resulting from a hybridization experiment performed by an independent contracting lab.

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Using a 0.8% TBE agarose gel, the following samples and controls were loaded:

- varying amounts (1000ng, 500ng, 250ng, 100ng, 50ng, and 10ng) of target A (*aad1*) PCR product
- varying amounts (500ng, 1000ng, 250ng, 100ng, 50ng, and 10ng) of target B (*aad12*) PCR product,
- two dilutions (1ng and 0.25ng) of the positive control tfdA PCR product DNA
- 500ng of the negative control pBR322 DNA (PCR product~850bp)
- 10µg of the negative control salmon sperm gDNA X Bam H1 digested
- 10µg of the negative control salmon sperm gDNA X Eco R1 digested
- 10µg of the negative control salmon sperm gDNA X Hind III

Invitrogen 1Kb+ DNA ladder was used for sizing

Standard S. blot transfer procedures for the turbo blotter (descending transfer) were employed.

The nylon filter was UV linked and prehybridized (10mM EDTA, 6 X SSC, 0.5% SDS, 10% formamide, 5 X Denhardt's solution, and 100ug/ml denatured sheared salmon sperm DNA) for 4 hours at 42 degrees C. with shaking.

The filter was hybridized using 20 ml of the same buffer used for the prehybridization solution and 1000ng of T4 PNK 5' end labeled ^{32}P tfdA DNA (total of 8,000,000dpm per 20ml) at 42 degrees C. for 24 hours.

The filter was rinsed at room temperature in 3 X SSC + 0.5% SDS for 5 minutes, followed by 2 rinses at room temperature (15 minutes each) in 3 X SSC + 0.1% SDS.

The **first wash** (3 changes) was carried out for **60 minutes** using **3 x SSC+ 0.1% SDS, at 35° C.**
The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1a)

The **second wash** (3 changes) was carried out for **2 hours** using **2 X SSC + 0.1% SDS at 68° C.**
The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1b)

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The **third wash** (3 changes) was carried out for **40 minutes** using **0.1 X SSC + 0.1% SDS at 68° C**. The filter was autoradiographed for 24 hours at -70°C. using an intensifier screen and Kodak BioMax MS film. (D1c).

The autoradiograph obtained after **first wash (low stringency; D1a)** showed negative control background with very strong positive control and target sample signal. The autoradiograph obtained after the **second wash (moderate stringency; D1b)** demonstrates that this moderate stringency wash removed nearly all of the negative control background and left strong positive control and target sample signal. The autoradiograph obtained after the **third wash (high stringency; D1c)** demonstrates that the third wash totally removed the negative control signal and left very strong positive control signal and good target sample signal even down to 10ng of both target DNAs.

"Target A (aad1)" corresponds to a nucleic acid encoding a protein of SEQ ID No. 9.

"TfdA" corresponds to a nucleic acid having the nucleotide sequence of tfdA gene of *Ralstonia eutropha* (Streber et al. 1987; D2).

The application at page 3, paragraph 11 indicates that "*TfdA catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction*". D3 (Fukumori and Hausinger) confirms the same. TfdA thus encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

The proteins encoded by TfdA gene and by the aad1 gene have 32% amino acid sequence identity (D4).

The experiments demonstrate that even under stringent hybridization conditions, the nucleotide sequence encoding SEQ ID No. 9 specifically hybridizes to a nucleic acid encoding a protein having 2,4 D enzymatic degrading activity (tfdA), which nucleic acid was known from the art.

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4. Added Subject Matter (Articles 76(1), 123(2) EPC and 100 (c))

The subject matter of the granted claims goes beyond the disclosure of the application as filed. The claims thus contravene the requirements of Article 123(2) EPC. It should be noted that the current divisional application as filed appears to be an exact copy of the parental application as filed. At that time no claims were filed, but the originally filed claims were copied as items in the description. Only on January 28, 2011, a set of claims to be pursued in the current application was filed. Accordingly, any subject matter identified as not being present in the divisional application will equally be subject matter extending beyond the scope of the originally filed application (Art 76(1) EPC and vice versa).

4.1 *Claim 1 contains subject matter not disclosed in the parent application as filed*

In his letter of January 28, 2011, the Patentee indicates originally filed claims 1, 6 and 8 as basis for newly submitted claim 1, together with paragraphs 126 and 127 as support for hybridization conditions.

Claim 1 as filed on January 28, 2011 and granted in unamended form, reads:

> *A transgenic plant cell comprising an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID No: 9, where said plant expresses said polynucleotide and produces said protein.*

Originally filed claim 1 reads:

> *A transgenic plant cell comprising* ***a polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO: 9 or a variant of SEQ ID NO: 9 wherein said variant has aryloxyalkanoate dioxygenase activity, at least one amino acid deletion or conserved substitution, and at least 95% sequence identity with SEQ ID NO: 9*** *(emphasis added).*

Originally filed claim 6 reads:

> *The plant of claim 5 wherein said herbicide is a phenoxy auxinic herbicide.*

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Originally filed claim 8 reads:

The plant of claim 5 wherein said herbicide is an aryloxyphenoxypropionate.

Originally filed claim 5 reads:

A transgenic plant comprising a plurality of cells according to claim 1, wherein expression of said polynucleotide renders said cells tolerant to an aryloxyalkanoate herbicide.

In other words, the transgenic plant of claim 1 as originally filed was limited to plants expressing AAD-1 or a highly related variant thereof having a particular enzymatic activity. In contrast, the transgenic plant cell of granted claim 1 extends to transgenic plant cells comprising any polynucleotide hybridizing "to any nucleotide molecule that encodes AAD-1" of any enzymatic activity which may degrade phenoxy auxin herbicides or aryloxyphenoxypropionate herbicide.

Paragraphs 126 and 127 merely describe hybridization conditions and indicate that a class of proteins can be defined by hybridization properties of the encoding polynucleotides, but does not make reference to the specific combination of characteristics now claimed.

Given the wide range of nucleic acids that can hybridize even under stringent conditions to the mentioned nucleotide sequence (see above), it will be clear that subject matter has been included in claim 1 which is not unambiguously and directly derivable from the application as originally filed and thus contravenes the requirements of Art 76(1).

4.2 *Claims 2-16 contain subject matter not disclosed in the parent application as filed*

Claims 2-16 are dependent upon claim 1. These claims therefore also contravene the requirements of Art 76(1). However, several dependent claims make further new combinations that cannot be unambiguously and directly derived from the parent application as filed.

E.g. claim 15 claims plants of claim 1 further comprising an insect-resistance gene derived from a number of listed organisms. The only basis to be found for insect-resistance gene is in originally filed claim 61. However, claim 61 refers to the plant as claimed in originally filed claim 5, i.e. transgenic plants with a much more circumscribed definition of the nucleotide sequence included in those transgenic plants than in the currently claimed transgenic plant cells.

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Claim 6 claims plant cells further comprising a glufosinate and a glyphosate tolerance trait. According to the Patentee, support for the subject matter of claim 3 can be found in paragraphs 16, 82, 98, 344, Table 25 and 346 (in Example 8). However, paragraph 16 mentions only that the invention includes "plants producing one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including but not limited to glyphosate, imidazolinone and glufosinate resistance genes". That is not a specific disclosure of plants further comprising only two specifically chosen tolerance genes from the list. Similarly, paragraph 82 lists even more herbicide tolerance genes which may be stacked, but does not single out specifically the further combination with glyphosate and glufosinate tolerance genes. Idem for paragraph 98. The other cited passages relate to Example 8, describing corn (maize) plants with specifically the AAD1(v3) transgene stacked with a glyphosate and glufosinate tolerance gene. However, this passage cannot provide the broad support required for current claim 3, claiming any plant with the combination of glufosinate and glyphosate tolerance traits with a transgene containing any polynucleotide hybridizing to any "nucleotide molecule that encodes AAD-1" of any enzymatic activity which may degrade phenoxy auxin herbicides or aryloxyphenoxypropionate herbicide.

4.3 *Claims 1 -16 contain subject matter not disclosed in the divisional application as filed*

As indicated above, any subject matter not present in the parent application as filed is necessarily also not present in the divisional application as filed, and the deficiencies identified above under Art 76(1) are also deficiencies under Art 123(2).

5. Priority entitlement (Article 87 EPC)

The claims as granted are not entitled to the benefit of the priority date of 30 April 2004 (filing date of US60/567,052 D7). Consequently, the relevant date for determining the prior art base is the international filing date, i.e. May 2, 2005.

Comparing the application as filed to the priority document, it will be immediately apparent that the priority application contains less teaching than the parent and divisional application as filed. In particular, no claims as originally filed in the parent application (and included as items in the divisional application) are included in the priority document, as well as a number of Sequences, Figures and Examples are missing from the priority document.

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Decision of the Enlarged Board of Appeal G2/98 has confirmed that an invention is only entitled to priority when the priority application clearly and unambiguously discloses that invention.

As set out above in connection with Art 100 (c), Art 76 and Art 123(2), the terms of the claims as granted cannot even be clearly and unambiguously derived from the parent or divisional application.

As the priority application contains even less teaching than the parent application or divisional application as filed, the subject matter of the claims can also not be derived clearly and unambiguously from the priority application as filed. The subject matter of these claims is therefore not entitled to any of the claimed priorities in accordance with G2/98.

Moreover, there are additional reasons why the claims are not entitled the benefit of the earlier priority dates.

As mentioned above, there are no claims included in the priority application. While some of the terms used in the current claims indeed can be found at various places in the application, there is no specific, clear and unambiguous disclosure of the combination of such features. Moreover, there are general terms which are only used once in the application as originally filed, exactly in the claims as filed, and therefore have no disclosure in the priority application.

E.g. the concept of "a protein that enzymatically degrades a herbicide selected from the group of a phenoxy auxin and an aryloxyphenoxyproprionate" as used in claim 1, can only be found in originally filed claim 47 in the context of an isolated polynucleotide sequence hybridizing with any one of three exactly defined nucleotide sequences encoding AAD1, and has no counterpart in the priority application nor can it be unambiguously derived therefrom. Claim 1 is thus not entitled to priority.

Claims 2-16 are dependent or refer to claim 1, and thus suffer from the same deficiency.

Additionally, the generic term of claim 14 (insect resistance gene derived from listed organisms) can only be found in claim 61 in the originally filed application and has no counterpart in the priority application.

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In conclusion, none of the subject matter of the claims can be derived clearly and unambiguously from the disclosure of the priority application.

The relevant date to establish the prior art base for all claims is therefore the international filing date of May 2, 2005. Consequently, documents D12 and D15 are prior art within the meaning of Art 54(2) EPC.

6 Lack of Novelty (Articles 54 EPC and 100(a))

The subject-matter of several of the granted claims lacks novelty over the documents which will be discussed in detail in the following sections.

a. Claims 1, 11, and 13 lack novelty over D8

D8 is a first document describing transgenic plants and plant cells expressing a bacterial detoxifying enzyme which plants and plant cells are resistant to 2,4-D herbicide. D8 also describes isolated polynucleotides based on a tfdA gene from the soil bacterium *Alcaligenes eutrophus*, now renamed as *Ralstonia eutropha*, JM134 under control of a CaMV 35S promoter (see page 811, right column, section entitled "Construction of plasmids for constitutive and light induced DPAM expression in plants"). Transgenic plants, and thus necessarily plant cells were grown in the greenhouse and sprayed with 2,4-D (page 813, right column, first paragraph). Expression of the DPAM gene in tissue culture in the presence of 2,4-D (page 813, right column, third paragraph).

i. *Claim 1 lacks novelty over D8.*

As indicated above (section 3.1.1.) the *tfda* coding region (D2; reference 9 in D8) hybridizes under stringent conditions with a nucleic acid encoding a protein of SEQ ID No. 9 (and thus necessarily also with the complement). *tfdA* has 2,4 D degrading enzymatic activity (application as filed, page 3, paragraph 11). 2,4D herbicide has been sprayed on the transgenic plants. Plants (and thus also the plant cells thereof) were tolerant to application of up to 10 kg/ha 2,4-D-Na (page 813, right column, first paragraph). The tolerance phenotype indicates that the plants and the cells thereof express the polynucleotide and produce the protein.

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D8 thus anticipates claim 1 by disclosing all features of the subject matter of claim 1.

ii. Claim 11 lacks novelty over D8.

Claim 11 further specifies the subject matter of claim 1 by requiring that the herbicide is a 2,4-D herbicide.

D8 discloses tolerance of the transgenic plants (and cells) to 2,4 D.

Claim 11 is thus anticipated by D8.

iii. Claim 13 lacks novelty over D8.

Claim 13 further defines the subject matter of claim 1 by specifying that the herbicide is selected from a group of herbicides, including 2,4-D.

D8 discloses tolerance of the transgenic plants (and cells thereof) to 2,4 D.

D8 therefore discloses transgenic plant cells as claimed in claim 13.

b. Claims 1, 11 and 13 lack novelty over D9

D9 is another document describing cloning of a tfdA gene under control of a CaMV35S promoter and expressing it in plant cells and plants, leading to 2,4 D tolerance. D9 discloses that the tfda region is operably linked to a CaMV35S promoter (page 535, left column, line 4), which is a promoter functional in plant cells. Leaf discs of the transgenic plants were assayed for their ability to shoot in the presence of 2,4-D herbicide (page 535, left column). Progeny of transgenic plants were germinated and grown for 4 to 8 weeks in soil, prior to spraying with 2,4 D herbicide (pages 538 left column, second section). 2,4D- tolerance was observed in transgenic plants and their progeny (and thus necessarily in the cells thereof).

D9 anticipates claims 1, 11 and 13 for the same reasons as explained under 6.a.

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c. ***Claims 1, 11 and 13 lack novelty over D10***

D10 is yet another publication wherein the tfdA gene of *A. eutrophus* JMP34 is cloned under the control of the CaMV 35S promoter (page 163, left column, second paragraph, lines 1 to 4). Transgenic cotton plants comprising this transgene are generated and sprayed with 2,4-D herbicide (page 167, left column, last paragraph; right column, end of the first paragraph). 2,4-D tolerant plants were obtained (see page 166, Figure 3 and page 168, Figure 4).

D10 thus anticipates claims 1, 11 and 13 for the same reasons as explained under 6.a.

d. ***Claims 1, 11 and 13 lack novelty over D11***

D11 is a publication wherein the tfdA gene of *A. eutrophus* is cloned under the control of the CaMV 35S promoter (page 645 right column, last paragraph; page 646, left column, lines 14 to 16). Transgenic tobacco and cotton plants were generated and sprayed with 2,4-D herbicide (see page 648, Fig 2; page 648 right column paragraph preceding the concluding remarks). Transgenic plants were tolerant to the herbicide (Fig 2 and Fig 4). Progeny plants were also scored for herbicide tolerance (page 648 right column, last paragraph of the section "Transgenic cotton plants").

D11 therefore also anticipates claims 1, 9, 12 and 17 as elaborated under 6.a.

e. ***Claims 1, 11 and 13 lack novelty over D12***

D12 is the last publication cited wherein the tfdA gene of *A. eutrophus* is cloned under the control of the CaMV 35S promoter (page 367 right column, third paragraph). Transgenic mustard plants were generated and sprayed in field trials with 2,4-D herbicide (page 368, right column, third paragraph and Table 2). Transgenic mustard plants and their progeny were tolerant to 2,4 D herbicide application (Fig 2, Table 2).

D12 therefore also anticipates claims 1, 11 and 13 as elaborated under 6.a.

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f. Conclusion

Claims 1 , 11 and 13 are anticipated by at least one of documents D8, D9, D10, D11 or D12.

7 **Inventive step (Articles 56 EPC and 100(b))**

a. Claims which are strictly speaking considered novel over any one of D8 to D12 lack an inventive step.

Claims 1, 11 and 13 are anticipated by one or more of documents D8 to D12. For the remaining claims, Opponent submits they lack an inventive step over the prior art.

i. *Claims 3, 5 -9, 12, 14 and 16 lack an inventive step over D8 (or any one of D9, D10, D11, D12) in combination with any one of D19, D20, D21, D22 or D23.*

Claims 3, 5-9, 12, 14 and 16 further define the transgenic plant cell of claim 1 by specifying that the plant cells may comprise other herbicide tolerance genes, particularly glufosinate tolerance genes, glyphosate tolerance genes, dicamba tolerance genes and genes conferring tolerance to ALS inhibitor herbicides and that tolerance to the corresponding herbicides may also be provided.

Documents D8 to D12 do not disclose such additional herbicide genes. However, the application does not claim any interaction between the presence of the phenoxy auxin herbicide or aryloxyphenoxypropionate herbicide degrading transgene and the other herbicide tolerance genes. The problems can thus be separated and merely amounts to the provision of plant cells with these other tolerance genes in addition to the problem of providing plant cells with the transgene as specified in claim 1.

It will be clear that these other tolerance genes, and their application for generating herbicide tolerant plant cells to confer tolerance to the corresponding herbicides is well known from the prior art, including D19 (glufosinate tolerance), D20 (glufosinate tolerance), D21 (glyphosate tolerance), D22 (dicamba tolerance) and D23 (ALS inhibitor tolerance).

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No inventive step can be derived from simple combination in a plant cell, of the transgene of claim 1 with the other herbicide tolerance genes.

ii. *Claims 2 and 10 lack an inventive step over D8 (or any one of D9, D10, D11, D12).*

Claims 2 and 10 merely define that the transgenic plant cell of claim 1 is a maize plant cell or a rice plant cell. No specific advantage is alleged for generating transgenic maize or rice plant cells, rather than cells of transgenic cotton, mustard, or tobacco plants and the choice of a particular plant cell cannot provide an inventive step.

iii. *Claim 4 lacks an inventive step over D8 (or any one of D9, D10, D11, D12) in combination with D15.*

D8 discloses transgenic plant cells comprising a tfdA encoding transgene, rather than the rdpA of SEQ ID No. 9, disclosed in the contested patent.

The technical problem can thus be formulated as providing transgenic plant cells with alternative phenoxy auxin or aryloxyphenoxyproprionate herbicide degrading enzyme encoding transgenes.

D15 discloses such coding regions involved in phenoxy auxin herbicide degradation. In fact, D15 discloses a coding region that is identical to SEQ ID No. 3 (except for an ambiguous Y nucleotide instead of C, see D13 and D14) and the encoded protein (SEQ ID No. 9) is identical.

A person skilled in the art would have substituted the tfdA gene in the polynucleotide of e.g. D8 for the coding region encoding the protein with enzymatic herbicide degrading capacity as disclosed in D15 to arrive at the claimed subject matter with reasonable expectation of success in view of the successes reported in any one of D8 to D12.

No inventive skill is needed to arrive at the subject matter of claim 4.

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iv. *Claim 15 lack an inventive step over D8 (or any one of D9, D10, D11, D12) in combination with D18.*

Claims 15 further defines the transgenic plant of claim 1 by specifying that the plants may also comprise other transgenes conferring interesting traits such as insect-resistance genes (claim 15).

Documents D8 to D12 do not disclose such additional transgenes. However, the application does not claim any interaction between the presence of the phenoxy auxin herbicide or aryloxyphenoxypropionate herbicide degrading transgene and the other transgenes. The problem can thus be separated into subproblems and merely amounts to the provision of plant cells with these other transgenes in addition to the problem of providing plant cells with the transgene as specified in claim 1.

It will be clear that these other transgenes, and their application in agronomy are well known from the prior art, including D18 (BT insect-resistance genes).

No inventive step can be derived from simply combining the transgene of claim 1 with other interesting transgenes known in the art in one plant cell.

8 Sufficiency of Disclosure (Articles 83 EPC and 100(b))

The invention as claimed in the patent as granted is insufficiently disclosed and therefore contravenes the requirements of Article 83 EPC.

The application provides support and disclosure only for a very limited number of methods and polynucleotides i.e. those having SEQ ID No. 3-5 which encode a polypeptide having SEQ ID No. 9 and transgenic plants comprising such polynucleotides.

As demonstrated above, the definition of the claimed polynucleotides by virtue of capacity to hybridize under stringent conditions, still encompasses a large genus of polynucleotides including polynucleotides encoding a protein having as little as 32% amino acid sequence identity, and is thus not congruent with the limited disclosure by the application.

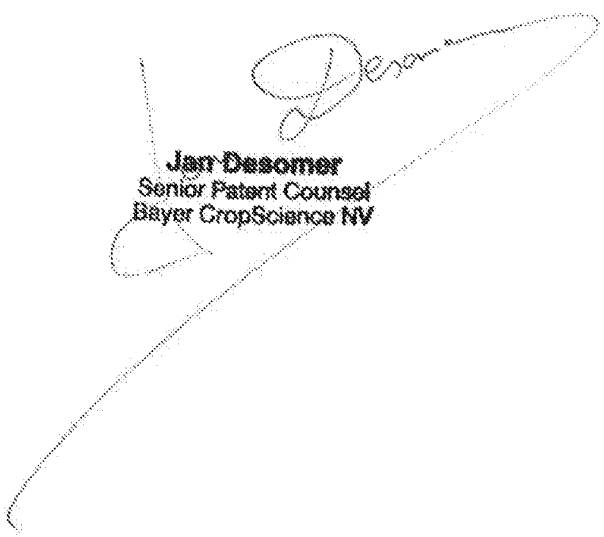
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Remark

Opponent further remarks that claim 4 of the current patent claims the same subject matter as claim 1 of EP 1 740 039 granted on the parent application. It is an accepted principle in most patent systems that two patents cannot be granted to the same applicant for one invention. The Enlarged Board of Appeal has accepted obiter dictum that the principle of the prohibition on double patenting is based on the notion that an applicant has no legitimate interest in proceedings leading to the grant of a second patent for the same subject-matter if he already possesses one granted patent for that subject-matter (see G 1/05, and G 1/06).

Conclusion

The subject matter of the claims as granted does not meet the requirements of the EPC. The patent should be revoked in its entirety.



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Conditions for Hybridization of Oligonucleotide Probes

When using oligonucleotides as probes, the aim is to find conditions that are stringent enough to guarantee specificity and sufficiently flexible to allow formation of stable hybrids at an acceptable rate. For DNA molecules more than 200 nucleotides in length, hybridization is usually carried out at 15–25°C below the calculated melting temperature (T_m) of a perfect hybrid. However, as the length of the probe is decreased, the T_m is lowered to the point where it is often impractical to carry out hybridization at T_m – 25°C. Typically, therefore, hybridization with synthetic oligonucleotides is carried out under conditions that are only 5–10°C below the T_m. Although such stringent conditions reduce the number of mismatched clones that are detected with short oligonucleotide probes, they have the less desirable consequence of reducing the rate at which perfect hybrids form.

Hybrids formed between DNA molecules more than 200 nucleotides in length are completely stable for all practical purposes. The chances that such a long stretch of double helix will unwind at temperatures 15–25°C below the T_m are extremely small. However, hybrids (even perfect hybrids) formed between short oligonucleotides and their target sequences at 5–10°C below the T_m are far easier to unwind, and hybridization reactions of this type can be regarded as reversible. This has important practical consequences. Whereas hybrids formed between longer DNA molecules are essentially stable under the conditions used for posthybridization washing, hybrids (even perfect hybrids) involving short oligonucleotides are not. Posthybridization washing of such hybrids must therefore be carried out rapidly so that the probe does not dissociate from its target sequence. For this reason, hybridizations with short oligonucleotides should be carried out under stringent conditions (5–10°C below the T_m) using high concentrations (0.1–1.0 pmole/ml) of probe. When only one or a small number of oligonucleotides (<8) are used as probes, the annealing reaction rapidly reaches equilibrium, and hybridization should therefore be terminated after 3 or 4 hours. More complex mixtures, in which the concentration of each oligonucleotide is comparatively low, require hybridization to be carried out for proportionately longer periods. For example, mixtures of 32 or more oligonucleotides should be hybridized for 1–2 days. Posthybridization washing should be carried out for brief periods of time, initially under conditions of low stringency and then under conditions of stringency equal to those used for hybridization.

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2

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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August 8, 2014
JZ/HE

Re.: European Patent No. 2 308 973 B1 (Application No. 10 012 208.1 - 1401)
Patent Proprietor: Dow AgroSciences LLC
Opposition by: Bayer CropScience NV
Our Ref.: 502-14 T2

This is in reply to the Opposition filed by Bayer CropScience NV on December 20, 2013 against EP 2 308 976 B1 and in response to the Communication of Notices of Opposition pursuant to Rule 79(1) EPC dated January 31, 2014.

1. Requests

1.1 It is requested that European Patent 2 308 976 B1 (in the following: the Opposed Patent) be maintained on the basis of amended claims 1 to 19 of the enclosed Main Request.

1.2 Oral proceedings according to Art. 116(1) EPC are requested in the event that the Opposition Division cannot agree to the above Main Request.

2. Amendments: Articles 76(1), 123(2) and 100(c) EPC

As acknowledged by the Opponent, the Opposed Patent was granted for a divisional application which was filed as an exact copy of the parent application as originally filed. The divisional application was not filed with a claim listing, but rather the originally filed claims were copied

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as items into the description. Consequently, any subject-matter that has a basis under Art. 76(1) EPC in the parent application as originally filed (see PCT publication WO 2005/107437) will at the same time also have a basis under Art. 123(2) EPC in the text of the divisional application as originally filed and *vice versa*.

The Opponent asserted that the subject-matter of claims 1, 17 and 18 contains subject-matter not disclosed in the parent application as filed, contrary to the provisions of Art. 76(1) EPC. Additional objections under Art. 76(1) EPC were raised against the subject-matter of claims 3, 14, and 15. Opponent's objections against the subject-matter of granted claims 1, 17 and 18 primarily relied on the assertion that the definition of AAD-1 in the claims of the parent application was narrower than in the claims allowed in this divisional application. This is of course no basis for a valid objection under Art. 76(1) EPC, since the parent and divisional applications as filed provide a broader definition of the gene and protein elsewhere in the application.

The subject-matter of the remaining claims (i.e. claims 2, 4-13 and 16) was also attacked under Art. 76(1) EPC, merely due to their dependence on claim 1. In addition, the Opponent asserted that all claims lacking a basis under Art. 76(1) EPC in the parent application would also lack a basis under Art. 123(2) EPC in the divisional application as filed.

The Patentee disagrees with these objections under Art. 76(1) and 123(2) EPC for the reasons outlined in the following. Since the Main Request filed herewith is based on the originally granted claims the objections raised by the opponents will be discussed in as far as features objected to are also comprised in the claims of the Main Request.

2.1 Claim 1 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.1.1 The Opponent asserted that the text passages indicated in Patentee's letter to the EPO of January 27, 2011, namely original claim 5, and paragraphs [00112], [00118], and [00124]-[00131] of the parent application, would not provide a basis for the subject-matter of granted claim 1, contrary to the provisions of Art. 76 EPC. The Opponent argued in particular that originally filed claim 5 "*was limited to plants expressing AAD-1 or a highly related variant thereof having a particular enzymatic activity*" (i.e., aryloxyalkanoate dioxygenase activity), while the transgenic plant of granted claim 1 encompasses "*transgenic plants comprising any polynucleotide hybridizing to any nucleotide molecule that encodes AAD-1 of any enzymatic activity which may degrade phenoxy auxin herbicides or aryloxyphenoxypropionate herbicides*" (see page 8, first paragraph of the Opposition brief). The Opponent further argued that paragraphs [00112], [00118], and [00124]-[00131] of the parent application, cited in the response of January 27, 2011, do not make reference to the specific combination of characteristics now claimed.

2.1.2 This analysis of the teaching of the parent application as originally filed is incorrect. Claim 5 of the parent application refers to claim 1, which characterize the variant as having *"arylalkanoate dioxygenase activity"*. The enzymatic activity recited in claim 1 is further described in paragraph [0020] of the parent application:

> *"This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides."* (Emphasis added)

This statement is also repeated in the first sentence of paragraph [0087] of the parent application.

Thus, the feature that the *"protein enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* is based on claims 1 and 5 and the more precise definition of the enzymatic activity in paragraphs [0020] and [0087] of the parent application.

2.1.3 The feature that *"said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9"* not only find basis in claim 5, which refers to claim 1 but also in the specification. For instance, paragraph [0149] of the parent application supports that said polynucleotide is comprised in a transgenic plant:

> *"The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants."*

and further in paragraph [0151] of the parent application:

> *"Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants."*

Thus, paragraphs [0149] and [0151] of the parent application disclose that the transgenic plants of the invention can comprise **any** polynucleotide encoding a protein of the invention.

The polynucleotides encoding proteins of the invention are further specified in paragraph [0095] of the parent application:

> *"In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides in vitro."*

It should particular be noted that the term *"genes and proteins of the invention"* does not only relate to the specifically disclosed polynucleotide and polypeptide sequences, as explained in paragraph [00135] of the parent application:

> *The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity.*

It is further set out in paragraph [0112] of the parent application:

> *"The subject invention uses classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide* ***by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence*** *(the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences."* (Emphasis added)

Accordingly, the parent application does not only disclose transgenic plants comprising polynucleotides fulfilling the features of claim 1 of the parent application as alleged by the Opponent but also plants comprising nucleotide sequences that are characterized by their ability to hybridize with a nucleotide sequence encoding an exemplified protein. One particularly preferred protein is the native AAD-1 of *Sphingobium herbicidovorans* according to SEQ ID NO: 9 (see paragraph [0050] of the parent application).

Furthermore, it is taught in paragraph [0126] of the parent application:

> *"Thus, the subject invention includes polynucleotides (and/or their complements,* ***preferably their full complements****) that hybridize with a polynucleotide exemplified herein. That is,* ***one way to define a gene*** *(and the protein it encodes), for example,* ***is by its ability to hybridize (under any of the conditions specifically disclosed herein)*** *with a known or specifically exemplified gene"* (Emphasis)

Furthermore it is taught in paragraphs [0124] and [0127] of the parent application that "stringent conditions" are preferred hybridization conditions, when characterizing the polynucleotides encoding the proteins of the invention.

Taken together the feature *"said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9"* finds basis in

paragraphs [0050], [0095], [0112] [0124], [0126], [0127], [00135], [00149] and [00151] as well as in claims 1 and 5 of the parent application.

2.1.4 Claim 1 of the Main Request has been further amended to characterize the protein encoded by the isolated polynucleotide present in the transgenic plant in **structural** terms, namely in that said protein is *"at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"*.

A basis for this structural characterization can be found in paragraphs [0045], [0046], [0047], and [0120] of the parent application which indicate SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5 to be polynucleotides encoding the most preferred proteins of the invention.

It is further taught in paragraph [0142] of the parent application

> *"Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that* ***the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins****. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein."* (Emphasis added)

Thus, the parent application also discloses polynucleotides encoding proteins having amino acid similarity to a protein encoded by SEQ ID NO: 3, SEQ ID NO: 4 or SEQ ID NO: 5. The value of 70% amino acid sequence identity is explicitly recited in paragraph [0142] of the parent application.

Taken together the feature *"wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"* finds direct and unambiguous basis in the parent application.

2.1.5 The parent application clearly provides direct and unambiguous basis for the subject-matter of claim 1 of the Main Request. Thus, the subject-matter of claim 1of the Main Request fulfills the requirements of Art. 76(1) EPC.

As explained above all paragraphs of the parent application as originally filed are identical to the paragraphs of the divisional application as originally filed and all claims of the parent application have been added on pages 159 to 168 of the divisional application. All subject-matter fulfilling the requirements of Art. 76(1) EPC as outlined in Sections 2.1.1 to 2.1.3, thus also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 1 is in accordance with the requirements of Articles 76(1) and 123(2) EPC.

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2.2 Claim 2 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.2.1 Claim 2 of the Main Request is based on granted claim 1, which has been amended to further characterize the protein encoded by the isolated polynucleotide present in the transgenic plant in **functional** terms, namely by specifying the degrading activity of said protein. More specifically, the previous feature:

"enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"

has been replaced by the following expression

"enzymatically degrades
(i) an R-enantiomer of chiral phenoxy auxin herbicide in addition to achiral phenoxy auxins,
(ii) an aryloxyphenoxypropionate herbicide, or
(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,"

2.2.2 The Opponent already acknowledged through the interpretation of the feature *"enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* (see page 6, paragraph 5 of the Opposition brief) that the feature of granted claim 1 encompasses the following three alternatives:

(1) "a phenoxy auxin herbicide"
(2) "an aryloxyphenoxypropionate herbicide"
(3) "a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"

Alternative (1) has been further specified in that the phenoxy auxin herbicide degrading activity is an activity that *"enzymatically degrades an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins"*. A literal basis for this feature can be found in paragraph [0074], fifth sentence, of the parent application.

All other features of granted claim 1 on which claim 2 is based find basis in the parent application as filed as explained in sections 2.1.2 and 2.1.3 above and, thus the subject-matter of claim 2 of the Main Request fulfils the requirements of Art. 76(1) EPC. All subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 2 of the Main Request is in accordance with the requirements of Art. 76(1) and 123(2) EPC.

2.3 Claim 4 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.3.1 The Opponent asserted that granted claim 3, which corresponds to claim 4 of the Main Request does not fulfil the requirements of Articles 76(1) and 123(2) EPC. The Opponent pointed out in particular that claim 3 defines the plant as comprising *"a glyphosate tolerance trait and a*

glufosinate tolerance trait". The Opponent argued that paragraph [0016] of the application as filed does not provide an adequate basis for these two tolerance traits. Allegedly, the recitation of only two tolerance traits, i.e. *"a glyphosate tolerance trait and a glufosinate tolerance trait"*, constitutes the singling-out of a specific combination of tolerance traits.

2.3.2 The Opponent's argument is flawed. Paragraph [0016] of the parent application teaches that:

> *"The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate, imidazolinone- and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options."* (Emphasis added)

Paragraph [0016] of the parent application, thus provides a list of three herbicide resistance genes, namely, glyphosate, imidazolinone-, and glufosinate-resistance genes. One of the preferred embodiments is deleted from the list of three elements. The deletion of one preferred embodiment from a list of three preferred embodiments is in agreement with the requirements of Art. 76(1) EPC in line with the principles set out in Enlarged Board of Appeal Decision G 2/10 regarding the permissibility of disclaiming an embodiment that is taught to belong to the invention under Art. 123(2) EPC. The deletion (disclaiming) of subject-matter taught as forming part of the invention is permissible, if the subject-matter remaining in the claim after the deletion of this element is directly and unambiguously disclosed (see Headnote 1b of G 2/10). This is clearly the case, when the embodiment "imidazolinone resistance" is removed from the list in paragraph [0016], since it is clear to the skilled person that in this case "glyphosate and glufosinate-resistance" remains. As set out above all subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 4 of the Main Request fulfils the requirements of Articles 76 (1) and 123(2) EPC.

2.4 Claims 15 and 16 of the Main Request fulfil the requirements of Art. 76(1) and Art. 123(2) EPC

2.4.1 The Opponent asserted that the subject-matter of granted claims 14 and 15, which correspond to claims 15 and 16 of the Main Request, contravenes the requirements of Articles 76(1) and 123(2) EPC.

The Opponent conceded that the wording of granted claims 14 and 15 can be found in the wording of original claims 61 and 62, respectively, of the parent application, but held that the granted claims do not comprise the limitations implied by the reference of these claims to original claims 5 and 1.

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2.4.2 Claims 61 refer to claim 5 of the parent application, which in turn refers to claim 1. As detailed in section 2.1 above, claims 1 and 5 together with paragraphs [0050], [0095], [0112], [0124], [0126], [0127], [0149] and [0151] of the parent application provide a basis under Art. 76(1) EPC for the subject-matter of claim 1 of the Main Request. Accordingly, the subject-matter of claim 15 of the Main Request finds basis under Art. 76(1) EPC in claims 1, 5 and 61 together with paragraphs [0050], [0095], [0112], [0124], [0126], [0127], [0149] and [0151] of the parent application.

2.4.3 In an analogous manner, claim 16 of the Main Request finds basis under Art. 76(1) EPC in paragraphs in claims 1, 5 and 62 together with paragraphs [0050], [0095], [0112] [0124], [0126], [0127], [0149] and [0151] of the parent application.

As explained in section 2 above, all subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claims 15 and 16 of the Main Request fulfils the requirements of Articles 76(1) and 123(2) EPC.

2.5 Claim 18 of the Main Request fulfils the requirements of Art. 76 and Art. 123(2) EPC

2.5.1 The Opponent asserted that the subject-matter of granted claim 17, which correspond to claim 18 of the Main Request, does not fulfil the requirements of Articles 76(1) and 123(2) EPC due to the reference to an *"asexual propagate"*. The Opponent noted that the term *"asexual propagate"* is only used once in the original specification, namely in originally filed claim 59, which is dependent on original claim 5.

The Opponent conceded, that the feature *"asexual propagate"* in granted claim 17 finds a basis under Art. 76(1) EPC in claim 59 of the parent application but held that the granted claim does not comprise the limitations implied by the reference of this claims to claims 5 and 1.

2.5.2 Original claim 59 is dependent on original claim 5, which is in turn dependent on original claim 1. As detailed in section 2.1 above, claims 1 and 5 together with paragraphs [0050], [0095], [0112] [0124], [0126], [0127], [00149] and [00151] of the parent application provide a basis under Art. 76(1) EPC for the subject-matter of claim 1 of the Main Request. Accordingly, the subject-matter of claim 18 of the Main Request finds a basis under Art. 76 EPC in claims 1, 5 and 59 together with paragraphs [0050], [0095], [0112], [0124], [0126], [0127], [0149] and [0151] of the parent application. As explained in section 2 above, all subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC.

2.6 Claim 19 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.6.1 The Opponent alleged that the subject-matter of granted claim 18, which corresponds to claim 19 of the Main Request, does not fulfil the requirements of Articles 76(1) and 123(2) EPC,

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because the text passages indicated as basis for this feature are part of the Example section and the particular combination of features recited in the Example section is not reflected in granted claim 18.

2.6.2 Claim 19 of the Main Request refers to claim 9 which in turn refers to claim 1 and 2 in the alternative. Through these dependencies, claim 19 is directed to ground tissue of a transgenic maize plant which comprises an isolated polynucleotide as defined in claim 1 or claim 2.

Paragraph [0320] of the parent application, which is numbered as subsection 7.10.1 describes methods for tissue harvesting, DNA isolation and quantification (see headline of this subsection). Subsection 7.10.1 is part of the bigger section 7.10 which is entitled *"Molecular Analysis: Maize Materials and Methods"* (see paragraph [00319] on page 92 of the original description). From the headings of sections 7.10 and 7.10.1, it is clear that paragraph [00320] refers to general methods for tissue harvesting, DNA isolation and quantification that can be carried out with any maize plant.

Paragraph [0320] describes that

> *Fresh tissue was placed into tubes and lyophilized at 4°C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill.*

The Opponent appears to be of the opinion that further features from this passage have to be included into granted claim 18, i.e. claim 19 of the Main Request, to fulfil the requirements of Art. 76(1) EPC and Art. 123(2) EPC. Opponent's position is not in line with the applicable case law of the Technical Boards of Appeal. A feature taught in the context of a specific embodiment/example may be separated from the remaining features in the specific embodiment/example under the condition that the extracted feature is not inextricably linked with the other features of the specific embodiment/example (see, e.g. T 714/00 Reasons for the decision No. 3.3). Thus, it has to be assessed whether the skilled person would have considered the feature *"grinding of maize"* to be inextricably linked to the other experimental parameters indicated, namely the use of a tungsten bead; grinding for 1 minute, the use of a Kelco bead mill, or lyophilisation for 2 d at 4°C.

The skilled person is aware that the grinding of maize tissue can be carried out with various apparatuses and is not limited to the specifically mentioned Kelco bead mill. Likewise, the skilled person will understand that maize tissue can be ground with beads made from different materials, with different numbers of beads, different length of grinding time and that the tissue can be lyophilized prior to grinding using various conditions. Therefore, none of the other features in the specific example in paragraph [0320] would be considered by the skilled person to be inextricably linked to the teaching that maize tissue is ground. Accordingly, paragraph [0320] of the parent

application provides a basis under Art. 76(1) EPC to specify that the tissue of the maize plant is ground. As explained in section 2 above, all subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC.

2.7 Claims 3, 5-14 and 17 of the Main Request fulfil the requirements of Art. 76(1) and Art. 123(2) EPC

2.7.1 The Opponent contended that the subject-matter of granted claims 2, 4-13 and 16, which correspond to claims 3, 5-14 and 17, respectively, of the Main Request, contravenes the requirements of Art. 76(1) EPC. However, the objections against granted claims 2, 4-13 and 16 were only based on the fact that said claims depend on claim 1.

2.7.2 Since the subject-matter of granted claim 1 as well as of claims 1 and 2 of the Main Request complies with the requirements of Art. 76(1) and Art. 123(2) EPC (see section 2.1 and 2.2 above), the objections against dependent claims 3, 5-14 and 17 of the Main Request are unfounded.

3. Entitlement to Priority, Art. 87 EPC

The Opponent held the view that the subject-matter of the granted claims is not entitled to the priority date of April 30, 2004 but only to the international filing date of May 2, 2005 and, therefore regarded **D12** (Bisht *et al.*, 2004) and **D15** (Schleinitz *et al.*, 2004) published after April 30, 2004 but prior to May 2, 2005 as prior art.

The Opponent cites **D12** in an attack on the novelty of the subject-matter of granted claims 1, 9, 12 and 17, which correspond to claims 1/2, 10, 13 and 18 of the Main Request, respectively. The Opponent further cited **D15** in an attack on the inventiveness of the subject-matter of granted claim 10, which corresponds to claim 11 of the Main Request.

Patentee does not regard it as expedient to discuss the validity of the priority claim for each and every claim of the Main Request. The only claims for which it may play a role whether they are entitled to the priority date of April 30, 2004 or not are claims 1, 2, 10, 11, 13 and 18 of the Main Request, which correspond to the claims attacked on the basis of **D12** and **D15** and, thus in the following analysis, it will only be discussed whether the subject-matter of claims 1, 2, 10, 11, 13, and 18 of the Main Request are entitled to the priority date of April 30, 2004.

3.1 Claim 1 of the Main Request is entitled to the priority date of April 30, 2004

The subject-matter of claim 1 of the Main Request has a basis in the priority document US 60/567,052 **D7**.

3.1.1 A basis for the feature "*A transgenic plant comprising an isolated polynucleotide that encodes a protein*" can be found at least in paragraph [00123] of the priority document (see page 29 of **D7**).

3.1.2 A basis for the feature "*that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide*" can be found at least in paragraph [0019] of the priority document (see page 6 of **D7**), especially in the first sentence of paragraph [0019].

3.1.3 A basis for the feature "*wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5*" can be found at least in paragraph [00116] of the priority document (see pages 27-28 of **D7**) in combination with paragraphs [0094], [0035], [0036] and [0037] of the priority document (see pages 7-8 of **D7**). The specific numerical value of 70% has a literal basis on page 28, line 2.

3.1.4 A basis for the feature "*wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9*" can be found at least in paragraphs [00100]-[00101] of the priority document (see page 22 of **D7**) in combination with paragraphs [0035] and [0039] of the priority document (see pages 7 and 8 of **D7**). It is explained in paragraph [00100] that

> "*[...] the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.*"

Stringent hybridization conditions are explained in the subsequent paragraph, i.e. in paragraph [00101] on pages 22-23 of **D7**.

One preferred protein sequence of the invention is the protein sequence of SEQ ID NO: 9 (see paragraph [0039]), which is the native amino acid sequence encoded by the *aad-1 (v1)* gene from *Sphingobium herbicidovorans* (see SEQ ID NO: 3 and the explanation in paragraph [0035]).

Taken together, the feature "*wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9*" has a clear basis in the priority document.

3.1.5 Finally, the feature *"wherein said plant expresses said polynucleotide and produces said protein"* has a basis at least in paragraph [00124] of **D7** (see page 29). Said paragraph is part of a section discussing transgenic hosts, especially transgenic plant hosts. The first sentence of paragraph [00124] reads:

> *In preferred embodiments, expression of the gene results, directly or indirectly in the intracellular production (and maintenance) of the protein(s) of interest.*

3.1.6 In conclusion, the subject-matter of claim 1 of the Main Request is fully based on the disclosure of the priority document and entitled to the priority date of April 30, 2004. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 1.

3.2 Claim 2 of the Main Request is entitled to the priority date of April 30, 2004

The subject-matter of claim 2 of the Main Request has a basis in the priority document US 60/567,052 **D7**.

3.2.1 Basis for the features:

- *"A transgenic plant comprising an isolated polynucleotide that encodes a protein"*;
- *"wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9"*, and
- *"wherein said plant expresses said polynucleotide and produces said protein"*

has already been provided in section 3.1 above.

3.2.2 A basis for the features *"that enzymatically degrades (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins, (ii) an aryloxyphenoxypropionate herbicide, or (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* can be found at least in paragraphs [0019] and [0051] of **D7** (see pages 6 and 9).

More specifically, paragraph [0019] of **D7** recites that

> *"This invention relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides."*

An almost identical statement can be found in paragraph [0061] of **D7**. Thus, paragraphs [0019] and [0061] provide a basis for the enzyme activities recited as alternatives (ii) and (iii) of claim 2.

Paragraph [0051] of **D7** teaches that

> *"An additional benefit of the AAD-1 gene is that unlike all fldA homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active*

isomers) of the chiral phenoxy auxins (e.g., dichlorprop and mecoprop) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid)."

This paragraph provides a basis for the enzyme activities recited in alternative (i) of claim 2.

3.2.3 In conclusion, the subject-matter of claim 2 of the Main Request is fully based on the disclosure in the priority document and entitled to the priority date of April 30, 2004. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 2.

3.3 Claim 10 of the Main Request is entitled to the priority date of April 30, 2004

There is ample support for the subject-matter of claim 10 in the priority document, e.g. in paragraph [0015], first sentence; and in paragraphs [0017], [0051], [0064], and [0074] of **D7**.

Accordingly, the subject-matter of claim 10 of the Main Request is fully based on the disclosure in the priority document and is entitled to the priority date of April 30, 2004. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 10.

3.4 Claim 11 of the Main Request is entitled to the priority date of April 30, 2004

3.4.1 Basis for the reference to SEQ ID NO: 9 in claim 11 can be found in paragraph [0040] of **D7** (see page 8). Basis for *"a variant of SEQ ID NO: 9"* can be found in paragraph [00109] of **D7** (see especially page 25, lines 1-3).

In said paragraph it is also indicated that variant proteins have equivalent or similar activity to an exemplified protein (see for example last sentence on page 24 of **D7**). This statement in combination with the information provided in paragraph [0019] provides a basis for the feature *"wherein said variant enzymatically degrades said herbicide"*.

3.4.2 Basis for the feature *"(has) at least one amino acid deletion or conserved substitution"* can be found in paragraph [00109] of the priority document.

3.4.3 Basis for the feature *"(has) at least 95% sequence identity with SEQ ID NO: 9"* can be found in paragraph [00116] of the priority document. The value of 95% has an explicit basis in the penultimate line of page 27 and in the third line of page 28. As already mentioned above, a basis for the reference to SEQ ID NO: 9 can be found in paragraph [0040] of the priority document (see page 8 of **D7**).

3.4.4 In conclusion, the subject-matter of claim 11 of the Main Request is fully based on the disclosure in the priority document and is entitled to the priority date of April 30, 2004. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 11.

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3.5 Claim 13 of the Main Request is entitled to the priority date of April 30, 2004

It is disclosed in paragraph [0019] that aryloxyalkanoate dioxygenase enzymes of the invention are *"capable of degrading phenoxy auxin and aryloxyphenoxypropionate (AOPP) herbicides"*. It is further disclosed in paragraph [0015] of the priority document that the *"subject invention provides novel plants that are not only resistant to 2,4-D, but also to AOPP herbicides"*. The abbreviation 2,4-D is explained in paragraph [0006] and refers to 2,4-dichlorophenoxyacetic acid. It is explained in paragraph [0014] that haloxyfop, quizalofop and diclofop are AOPP herbicides. It is further indicated in paragraph [0051] that MCPA, dichlorprop, and mecoprop are phenoxy auxin herbicides (see page 9, lines 5-8 of **D7**). As mentioned in paragraph [00243], quizalofop, cyhalofop and haloxyfop are AOPP herbicides (see paragraph [00243], especially page 64, lines 6-8 of **D7**). Finally, paragraph [00313] of **D7** contains the information that fluazifop, haloxyfop, quizalofop, and fenoxaprop are AOPP herbicides.

Accordingly, the herbicides listed have a basis in the priority document and are entitled to the priority date of April 30, 2004. Therefore, **D12** and **D15** do not constitute prior art for these herbicides.

3.6 Claim 18 of the Main Request is entitled to the priority date of April 30, 2004

A basis for plant tissues, parts and seeds referred to in claim 18 of the Main Request can be found in paragraph [00125] of the priority document. A basis for progeny can be found in paragraph [00130] of the priority document.

In summary, the subject-matter of claim 18 of the Main Request is based on the disclosure in the priority document and is entitled to the priority date of April 30, 2004. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 18.

3.7 Summarizing, at least the subject-matter of granted claims 1, 2, 10, 11, 13 and 18 of the Main Request is entitled to the priority date of April 30, 2004. Accordingly, **D12** and **D15** do not form prior art for the subject-matter of these claims.

4. **Novelty: Articles 54 and 100(a) EPC**

The Opponent raised novelty objections against the subject-matter of granted claims 1, 9, 12 and 17, which correspond to claims 1/2, 10, 13 and 18 of the Main Request, respectively. The subject-matter of these claims was said to be anticipated by each one of **D8**, **D9**, **D10**, **D11**, and **D12**, respectively.

These five novelty attacks are based on an identical line of argumentation, since all of **D8** to **D12** refer to transgenic dicot plants comprising the *tfdA* gene from *Ralstonia eutropha* (previous name: *Alcaligenes eutrophus*; later renamed as: *Wautersia eutropha*; current name: *Cupriavidus necator*). The Opponent has submitted results of experiments as **D1** which allegedly demonstrate that the *tfdA* gene hybridizes under "stringent conditions" with the polynucleotide recited in claim 1. The Opponent further pointed out that the TfdA protein catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction and that TfdA protein degrades a phenoxy auxin herbicide as required in one alternative by granted claim 1.

In the enclosed Main Request claim 1 was split into two independent claims. In amended claim 1, the encoded protein is further defined in structural terms. In amended claim 2, the encoded protein is further defined in functional terms.

4.1 The subject-matter of amended claim 1 is novel over the prior art

4.1.1 Without acquiescing to Opponent's assertion that the results of the experiments submitted as **D1** actually show that polynucleotides encoding TfdA hybridizes **under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9** Patentee has decided to address this objection by amendment. Accordingly, amended claim 1 further characterizes the protein encoded by the isolated polynucleotide present within the transgenic plant in that it is

> *"at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"*

4.1.2 The Opponent has carried out a sequence alignment between the protein encoded by the *tfdA* gene of *Ralstonia eutropha* and the *aad-1* gene of the Opposed Patent (see **D4**). According to this sequence alignment the protein encoded by the *tfdA* gene of *Ralstonia eutropha* exhibits about 32% sequence identity to the protein encoded by the *aad-1* gene of the Opposed Patent. The polynucleotide sequence according to SEQ ID NO: 3 encodes the wild type AAD-1 protein according to SEQ ID NO: 9. The polynucleotide sequence according to SEQ ID NO: 4 encodes the AAD-1 protein according to SEQ ID NO: 10, which only differs from wild type AAD-1 in an Arg to Cys mutation at position 212 and a His to Ala mutation at position 2. The polynucleotide sequence according to SEQ ID NO: 5 encodes the AAD-1 protein according to SEQ ID NO: 11, which only differs from wild type AAD-1 protein by an additional Ala that is inserted between the N-terminal Met and the His at position 2. In its alignment Opponent appears to have used the wild type AAD-1 amino acid sequence according to SEQ ID NO: 9 (it is noted that position 212 of the query sequence is Arg and not Cys and that position 15 is Ile which is only the case in the amino acid sequence according to SEQ ID NO: 9). Accordingly,

the alignment with either the protein according to SEQ ID NO: 10 or 11 will lead to an even lower level of sequence identity than 32%.

4.1.3 Claim 1 of the Main Request requires that the transgenic plant comprises a polynucleotide encoding a protein that is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, i.e. the proteins with the amino acid sequences according to SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11, respectively. As explained above, the protein encoded by the *tfdA* gene from *Ralstonia eutropha* exhibits 32% or less sequence identity to the amino acid sequences of SEQ ID NO: 9, 10 or 11. Therefore, the transgenic plants described in **D8**, **D9**, **D10**, **D11**, and **D12** do not anticipate the subject-matter of amended claim 1 and hence the subject-matter of claim 1 of the Main Request is novel over the prior art.

For the sake of completeness, it is noted again that **D12** does not even constitute prior art for the subject-matter of amended claim 1 of the Main Request (see section 3.1 above).

4.2 The subject-matter of amended claim 2 is novel over the prior art

4.2.1 Amended claim 2 characterizes the protein encoded by the isolated polynucleotide present within the transgenic plant in that it

enzymatically degrades
(i) an R-enantiomer of chiral phenoxy auxin herbicide in addition to achiral phenoxy auxins,
(ii) an aryloxyphenoxypropionate herbicide, or
(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,

4.2.2 Documents **D8**, **D9**, **D10**, **D11**, and **D12** cited by the Opponent as relevant for novelty relate to transgenic plants comprising the *tfdA* gene from *Ralstonia eutropha*. The TfdA protein encoded by said gene is described as possessing achiral phenoxy auxins, i.e. 2,4-D, degrading activity. The TfdA protein cannot enzymatically degrade R-enantiomers of chiral phenoxy auxin herbicides as required by alternative (i) of amended claim 2. Further, the TfdA protein is not capable of degrading aryloxyphenoxypropionate herbicides as required by alternatives (ii) and (iii) of amended claim 2.

4.2.3 In summary: the protein encoded by the *tfdA* gene of *Ralstonia eutropha* is described as possessing achiral phenoxy auxin degrading activity in **D8**, **D9**, **D10**, **D11**, and **D12**. Moreover, the *tfdA* gene of *Ralstonia eutropha* does not possess the enzymatic activities recited in alternatives (i), (ii) and (iii) of amended claim 2. Therefore, the transgenic plants disclosed in **D8**, **D9**, **D10**, **D11**, and **D12** do not anticipate the subject-matter of amended claim 2. Hence the subject-matter of amended claim 2 is novel over the prior art.

For the sake of completeness, we would like to repeat that **D12** does not even constitute prior art for the subject-matter of amended claim 2 of the Main Request (see section 3.2 above).

4.3 The subject-matter of amended claims 3 to 19 is novel over the prior art

Amended claims 3 to 19 of the Main Request are directly or indirectly dependent on claim 1 or 2. Accordingly, the subject-matter of claims 3 to 19 derives its novelty from this dependency.

5. Inventive Step; Articles 56 and 100(a) EPC

5.1 The Opponent has alleged that claims 1, 9, 12 and 17 as granted are anticipated by one or more of documents **D8** to **D12**, all of which are concerned with the known 2,4-D-herbicide degrading enzyme TfdA. An objection of lack of inventive step is raised against the subject-matter of granted claims 2-7, 11 and 13 based upon **D8** (or any one of **D9**, **D10**, **D11**, or **D12**), when considered in combination with **D19** (or any one of **D20**, **D21** or **D22**); against the subject-matter of granted claim 8 based upon **D8** (or any one of **D9**, **D10**, **D11**, or **D12**) taken alone; against the subject-matter of granted claim 10 based upon **D8** (or any one of **D9**, **D10**, **D11**, or **D12**), when considered in combination with **D15**; against the subject-matter of granted claims 14 and 15 based upon **D8** (or any one of **D9**, **D10**, **D11**, or **D12**), when considered in combination with **D18** or **D24**, respectively; and against the subject-matter of granted claim 18 based upon **D8** (or any one of **D9**, **D10**, **D11**, or **D12**) taken alone.

5.2 In this respect the Patentee reiterates that the claims of the Main Request can be considered entitled to the priority date of April 30, 2004 and thus **D12** and **D15** are not citable prior art.

Further, while not acquiescing to the Opponent's objection of lack of novelty over **D8** to **D11**, the independent claims of the Main Request presented herewith are amended to clearly recite additional features not disclosed in those documents.

For example, claim 1 now includes the feature that the encoded protein:

> *"is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5".*

Claim 2 now includes the feature that the encoded protein enzymatically degrades

> *(i) an R-enantiomer of chiral phenoxy auxin herbicide in addition to achiral phenoxy auxins;*
> *(ii) an aryloxyphenoxypropionate herbicide; or*
> *(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.*

It is submitted that claims 1 to 19 of the Main Request are both novel and inventive over all of the art cited by the Opponent and in the absence of any submissions to the contrary, the Patentee makes no further comments at this time. However, the Patentee reserves the right to make further submissions concerning the inventive step of the Main Request as necessary.

6. Sufficiency of Disclosure; Articles 83 and 100(b) EPC

6.1 The Opponent asserted that the invention as claimed in the granted claims is insufficiently disclosed, contrary to the provisions of Art. 83 EPC. The Opponent held the view that the specification provides support and disclosure only for polynucleotides having SEQ ID NOs: 3 to 5 but not for the broad definition based on stringent hybridization which allegedly covers polynucleotides having as little as 32% sequence identity to SEQ ID NO: 9. Allegedly, the broad scope of the granted claims is not congruent with the limited disclosure of the application.

6.2 The enclosed Main Request contains two independent claims that are each based on claim 1 as granted. Amended claim 1 is further limited in structural terms and amended claim 2 is further limited in functional terms (see sections 2.1 and 2.2 above). In view of these limitations, the Opponent's objections under Art. 83 EPC are moot.

7. Double Patenting

7.1 In a remark on page 18 of the Opposition Brief, the Opponent alleged that granted claim 10, which corresponds to claim 11 of the Main Request claims the same subject-matter as claim 5 granted in the parent patent EP 1 740 039 B1.

7.2 According to Art. 100 EPC, an opposition may only be filed on the grounds recited in paragraphs (a), (b) and (c) of Art. 100 EPC. In other words, the list of grounds for opposition in Art. 100 EPC is exhaustive. Double patenting is not listed as a ground for opposition and can, thus not be a basis to revoke this patent (see for example T 936/04). Consequently, Patentee sees no reason to further address Opponent's allegation.

8. Summary

From the above discussion it is evident that the subject-matter claimed in the Main Request fulfils the requirements of Art. 54, 56, 83, 76(1), and 123(2) EPC.

Therefore, our request to maintain the Opposed Patent on the basis of the Main Request is fully justified.

Dr. Jörk Zwicker
European Patent Attorney

Enclosures:
Main Request (marked-up copy and as clean copy)

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July 28, 2014
JZ/HE

Re.: **European Patent No. 2 298 901 B1 (Application No. 10 012 201.9 - 1401)**
Patent Proprietor: **Dow AgroSciences LLC**
Opposition by: **Bayer CropScience NV**
Our Ref.: **502-14 T3**

This is in reply to the Opposition filed by Bayer CropScience NV of December 6, 2013 against EP 2 298 901 B1 and in response to the Communication of Notices of Opposition pursuant to Rule 79(1) EPC dated January 16, 2014.

1. Requests

1.1 It is requested that European Patent 2 298 901 B1 (in the following letter: the Opposed Patent) be maintained on the basis of amended claims 1 to 11 of the enclosed Main Request.

1.2 Oral proceedings according to Art. 116(1) EPC are requested in the event that the Opposition Division cannot agree to the above Main Request.

2. Amendments: Articles 76(1), 123(2) and 100(c) EPC

As acknowledged by the Opponent the Opposed Patent was granted for a divisional application which was filed as an exact copy of the parent application as originally filed. The divisional application was not filed with claim listing rather the originally filed claims were copied as items into the description. Consequently, any subject-matter that has a basis under Art. 76(1)

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EPC in the parent application as originally filed (see PCT publication WO 2005/107437) will at the same time also have a basis under Art. 123(2) EPC in the text of the divisional application as originally filed and *vice versa*.

The Opponent asserted that the subject-matter of claim 1 contains subject-matter not disclosed in the parent application as filed, contrary to the provisions of Art. 76(1) EPC. Additional objections under Art. 76(1) EPC were raised against the subject-matter of claims 2 and 4. Opponent's objections against the subject-matter of granted claim 1 primarily relied on the assertion that the definition of AAD-1 in the claims of the parent application was narrower than in the claims allowed in this divisional application. This is of course no basis for a valid objection under Art. 76(1) EPC since the parent and divisional applications as filed provide a broader definition of the gene and protein elsewhere in the application.

The subject-matter of the remaining claims (i.e. claims 3 and 5-10) was also attacked under Art. 76(1) EPC, merely due to their dependence on claim 1. In addition, the Opponent asserted that all claims lacking a basis under Art. 76(1) EPC in the parent application would also lack a basis under Art. 123(2) EPC in the divisional application as filed.

The Patentee disagrees with these objections under Art. 76(1) and 123(2) EPC for the reasons outlined in the following paragraphs. Since the Main Request filed herewith is based on the originally granted claims the objections raised by the opponents will be discussed in as far as features objected to are also comprised in the claims of the Main Request.

2.1 Claim 1 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.1.1 The Opponent argued that granted claim 1 contains the functional definition *"having alpha-ketoglutarate-dependent dioxygenase activity"*, while originally filed claim 47 characterized the protein encoded by the claimed polynucleotide by a different functional definition, namely in that the protein *"enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and aryloxyphenoxypropionate"*.

In the Opponent's view, this exchange in the functional characterization of the encoded protein lacks a basis under Art. 76(1) EPC in the parent application as originally filed and a basis under Art. 123(2) EPC in the present application as originally filed.

Without acquiescing to Opponent's above assertions, Patentee has decided to address this objection by amendment. In particular, the functional characterization *"enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate herbicide"* is once again introduced into claim 1 of the Main Request so that amended claim 1 contains **both** functional characterizations.

Because of the re-introduction of the original functional definition, the Opponent's objections under Art. 76(1) EPC and Art. 123(2) no longer apply.

For the sake of completeness, we would like to note that the feature *"having alpha-ketoglutarate-dependent dioxygenase activity"* recited in granted claim 1 and in amended claim 1 of the Main Request finds a basis under Art. 76 EPC at least in paragraphs [0018], [0085] and [0086] (see page 5, lines 4 to 2 from the bottom; page 17, lines 7 to 10; page 17, lines 16 to 18, respectively).

2.1.2 Claim 1 of the Main Request has been further amended to characterize the protein encoded by the isolated polynucleotide present in the transgenic plant in **structural** terms, namely in that said protein is *"at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"*.

A basis for this structural characterization can be found in paragraphs [0045], [0046], [0047], and [0120] of the parent application which indicate SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5 to be polynucleotides encoding the most preferred proteins of the invention. In addition, it should be noted that claim 1 as granted, which is based on original claim 47 of the parent application, already makes reference to SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5.

It is further taught in paragraph [0142] of the parent application

> *"Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that* ***the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins****. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein."* (Emphasis added)

Thus, the parent application also discloses polynucleotides encoding proteins having amino acid similarity to a protein encoded by SEQ ID NO: 3, SEQ ID NO: 4 or SEQ ID NO: 5. The value of 70% amino acid sequence identity is explicitly recited in paragraph [0142] of the parent application.

Taken together the feature *"wherein said protein is at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"* finds direct and unambiguous basis in the parent application.

2.1.3 The parent application clearly provides direct and unambiguous basis for the subject-matter of claim 1 of the Main Request. Thus, the subject-matter of claim 1 of the Main Request fulfills the requirements of Art. 76(1) EPC.

As explained above all paragraphs of the parent application as originally filed are identical to the paragraphs of the divisional application as originally filed and all claims of the parent application have been added on pages 159 to 168 of the divisional application. All subject-matter fulfilling the requirements of Art. 76(1) EPC as outlined in Sections 2.1.1 to 2.1.2, thus also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 1 is in accordance with the requirements of Articles 76(1) and 123(2) EPC.

2.2 Claim 2 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

Claim 2 of the Main Request is based on granted claim 1, which has been amended to further characterize the protein encoded by the isolated polynucleotide present in the transgenic plant in **functional** terms, namely by specifying the degrading activity of said protein. In particular, the feature re-introduced into amended claim 1 (see section 2.1.1 above):

"enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"

has been further limited to the following functional characterization:

"enzymatically degrades
(i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
(ii) an aryloxyphenoxypropionate herbicide, or
(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,"

More specifically, the feature *"a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* recited in claim 47 originally filed in the parent application encompasses the following three alternatives:

(1) "a phenoxy auxin herbicide"
(2) "an aryloxyphenoxypropionate herbicide"
(3) "a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"

In the enclosed amended claim 2, alternative (1) has been further specified in that the phenoxy auxin herbicide degrading activity is an activity that *"enzymatically degrades an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins"*. A literal basis for this feature can be found in paragraph [0074], fifth sentence, of the parent application.

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Alternative (2) and alternative (3) find basis in originally filed claim 47 and alternative (3) further in paragraph [0020], first sentence and paragraph [0087], first sentence.

All other features of granted claim 1, on which claim 2 is based, find basis in the parent application as filed as explained above in section 2.1.1 and, thus the subject-matter of claim 2 of the Main Request fulfils the requirements of Art. 76(1) EPC. All subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 2 of the Main Request is in accordance with the requirements of Art. 76(1) and 123(2) EPC.

2.3 Claim 3 and 5 of the Main Request fulfil the requirements of Art. 76(1) and Art. 123(2) EPC

The Opponent asserted that the subject-matter of granted claims 2 and 4, which correspond to claims 3 and 5 of the Main Request, contravenes the requirements of Articles 76(1) and 123(2) EPC.

The Opponent noted that the wording of granted claims 2 and 4 finds a basis in the wording of claims 45 and 46, respectively, of the parent application, but noted that claims 45 and 46 contain a limitation (through their dependency on claim 44 of the parent application) that is not present in granted claims 2 and 4. More specifically, the Opponent asserted that the feature *"having aryloxyalkanoate dioxygenase activity"* is present in claims 45 and 46 of the parent application but is allegedly absent in granted claims 2 and 4.

Granted claims 2 and 4 have been renumbered as claims 3 and 5, respectively, in the enclosed Main Request. Thus, the above objection pertains to claims 3 and 5 of the Main Request. Claims 3 and 5 are dependent on claims 1 and 2.

Patentee disagrees with the Opponent's point of view. Claim 1 and claim 2 both require that the encoded protein *"enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"*. As explained above (see sections 2.1.1 and 2.2), this feature is literally recited in claim 1 and is also present in claim 2 but with a further limitation. As will be explained below, this enzymatic activity necessarily requires that the protein has aryloxyalkanoate dioxygenase activity. Accordingly, the feature, *"having aryloxyalkanoate dioxygenase activity"* is present in claims 3 and 5 due to the dependency on claims 1 and/or 2.

More specifically, it is disclosed in paragraph [0015] of the specification (see page 4 of the parent application as originally filed) that

> *Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxy auxins (such as 2,4-D and*

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> *dichlorprop), pyridyloxy auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac).* (Emphasis added)

From this passage it is clear that aryloxyalkanoate is a more generic term that encompasses phenoxy auxins and aryloxyphenoxypropionates. It is further explained in paragraph [0020] of the specification (see page 6 of the parent application as originally filed) that

> *This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides.* (Emphasis added)

Also from this passage it is clear that aryloxyalkanoate dioxygenase is a more generic term that encompasses enzymes capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. In other words, there may be aryloxyalkanoate dioxygenases that degrade herbicides other than phenoxy auxins and aryloxyphenoxypropionates. However, any enzyme capable of degrading phenoxy auxins and aryloxyphenoxypropionates is an aryloxyalkanoate dioxygenase.

Both claim 1 and claim 2 require that the protein encoded by the claimed polynucleotide enzymatically degrades a phenoxy auxin and/or an aryloxyphenoxypropionate. It is further required in claims 1 and 2 that the protein encoded by the claimed polynucleotide has (alpha-ketoglutarate dependent) dioxygenase activity. Accordingly, the protein encoded by the polypeptide of claim 1 or 2 has aryloxyalkanoate dioxygenase activity. Since claims 3 and 5 are dependent on claims 1 and/or 2, the proteins encoded by the polypeptides of claim 3 and 5 have aryloxyalkanoate dioxygenase activity, too.

Summarizing the above, the subject-matter of claims 3 and 5 of the Main Request finds a basis within the meaning of Art. 76(1) EPC in the parent application as originally filed. As explained in section 2.1 above, all subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claims 3 and 5 of the Main Request fulfils the requirements of Articles 76(1) and 123(2) EPC.

2.4 Claims 4 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

The Opponent contended that the subject-matter of granted claim 3, which corresponds to claim 4 of the Main Request, contravenes the requirements of Art. 76(1) EPC. However, the objection against granted claim 3 was only based on the fact that said claim depends on claim 1.

Since the subject-matter of granted claim 1 as well as the subject-matter of claims 1 and 2 of the Main Request complies with the requirements of Art. 76(1) and Art. 123(2) EPC (see sections

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2.1 and 2.2 above), the Opponent's objections against dependent claim 4 of the Main Request is unfounded.

Furthermore, claim 4 in the enclosed Main Request has been amended by re-defining the enzymatic activity of the encoded protein. More specifically, the characterization of the protein as *"having R-specific alpha-ketoglutarate-dependent dioxygenase activity relative to a 2,4 D-herbicide"* has been amended to read *"having R-specific alpha-ketoglutarate-dependent dioxygenase activity relative to chiral phenoxy auxins"*.

A basis for this functional characterization of the enzymatic activity can be found in paragraph [0074] of the priority document (see particularly the fifth sentence of paragraph [0074] on page 10 of the parent application).

Summarizing the above, the subject-matter of claim 4 of the Main Request finds a basis within the meaning of Art. 76(1) EPC in the parent application as originally filed. As explained in section 2.1 above, all subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 4 of the Main Request fulfils the requirements of Articles 76(1) and 123(2) EPC.

2.5 Claims 6-11 of the Main Request fulfil the requirements of Art. 76(1) and Art. 123(2) EPC

The Opponent contended that the subject-matter of granted claims 5-10, which correspond to claims 6-11, respectively, of the Main Request, contravenes the requirements of Art. 76(1) EPC. However, the objections against granted claims 5-10 were only based on the fact that said claims depend on claim 1.

Since the subject-matter of granted claim 1 as well as the subject-matter of claims 1 and 2 of the Main Request complies with the requirements of Art. 76(1) and Art. 123(2) EPC (see sections 2.1 and 2.2 above), the objections against dependent claims 6-11 of the Main Request are unfounded.

3. Entitlement to Priority, Art. 87 EPC

The Opponent held the view that the subject-matter of the granted claims is not entitled to the priority date of April 30, 2004 but only to the international filing date of May 2, 2005 and, therefore regarded **D12** (Bisht *et al.*, 2004) and **D15** (Schleimtz *et al.*, 2004) published after April 30, 2004 but prior to May 2, 2005 as prior art.

The Opponent cites **D12** in an attack on the novelty of the subject-matter of granted claims 1, 5, 7 and 9, which correspond to claims 1, 2, 6, 8 and 10 of the Main Request. The Opponent further cited **D15** in an attack on the inventiveness of the subject-matter of granted claim 3, which corresponds to claim 4 of the Main Request.

Patentee does not regard it as expedient to discuss the validity of the priority claim for each and every claim of the Main Request. The only claims for which it may play a role whether they are entitled to the priority date of April 30, 2004 or not are claims 1, 2, 4, 6, 8, and 10 of the Main Request, which correspond to the claims attacked on the basis of **D12** and **D15** and, thus in the following analysis, it will only be discussed whether the subject-matter of claims 1, 2, 4, 6, 8, and 10 of the Main Request are entitled to the priority date of April 30, 2004.

3.1 Claim 1 of the Main Request is entitled to the priority date of April 30, 2004

The subject-matter of claim 1 of the Main Request has a basis in the priority document US 60/567,052 **D7.**

3.1.1 Several passages of the priority document explain that the subject invention is directed to isolated polynucleotides encoding proteins of the subject invention. The priority document even contains a chapter entitled "Polynucleotides and Probes" (see paragraphs [0092] to [00106] on pages 19 to 23 of **D7**, see especially the title and the first sentence of paragraph [0092]).

A basis for the feature "*An isolated polynucleotide that encodes a protein having alpha-ketoglutarate-dependent dioxygenase activity*" can be found at least in paragraph [0059] of the priority document (see page 11 of **D7**; especially the last two lines on page 11). In this sentence it is explained that the genes of the subject invention can be classified in the family of alpha-ketoglutarate-dependent dioxygenases.

3.1.2 A basis for the feature "*wherein said protein enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide*" can be found at least in paragraph [0019] of the priority document (see page 6 of **D7**), especially in the first sentence of paragraph [0019].

3.1.3 A basis for the feature "*wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 3 and SEQ ID NO: 4*" can be found at least in paragraphs [00100]-[00101] of the priority document (see page 22 of **D7**) in combination with paragraphs [0035]-[0037] of the priority document (see pages 7 and 8 of **D7**). It is explained in paragraph [00100] that

> *"[...] the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene."*

Stringent hybridization conditions are explained in the subsequent paragraph, i.e. in paragraph [00101] on pages 22-23 of **D7**.

Three particularly preferred nucleotide sequences of the invention are the nucleotide sequences of SEQ ID NO: 3, 4, and 5 (see paragraphs [0035]-[0037]), which are the native nucleotide sequence of *AAD-1 (v1)* from *Sphingobium herbicidovorans*; the modified sequence *AAD-1 (v2)* in which an internal *Not* I site has been removed; and the plant-optimized sequence *AAD-1 (v3)*, respectively.

The Opponent asserted that SEQ ID NO: 5 disclosed in the priority document is not identical to SEQ ID NO: 5 disclosed in the Opposed Patent as originally filed. Allegedly, the 3' ends would differ between the two sequences. We disagree with this allegation. The last five pages of the priority document contain sequence information. SEQ ID NO: 5 is shown on the second page of these five pages. The opposed patent is a divisional application, and the sequence listing for the present divisional application was filed with the EPO on September 30, 2010. This originally filed sequence listing contains nineteen pages. SEQ ID NO: 5 is shown on page 3 of the sequence listing and is identical to SEQ ID NO: 5 of the priority document. In particular, contrary to the Opponent's assertions, both sequences have the same 3' end, namely GCTTAATCAC CTAGAGCTC.

Summarizing the above, the feature *"wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 3 and SEQ ID NO: 4"* has a clear basis in the priority document.

3.1.4 A basis for the feature *"wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"* can be found at least in paragraph [00116] of the priority document (see pages 27-28 of **D7**) in combination with paragraphs [0035], [0036] and [0037] of the priority document (see pages 7-8 of **D7**).

As already explained above in section 3.1.3, the nucleotide sequences of SEQ ID NO: 3, 4, and 5 (see paragraphs [0035]-[0037], and [0094]) and the protein encoded by these sequences are particularly preferred sequences of the subject invention.

It is explained in paragraph [00116] of the priority document that

> *Preferred polynucleotides and proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges.*

The specific numerical value of 70% has a literal basis within paragraph [00116] of the priority document **D7**, namely on page 28, line 2.

Taken together, the feature *"wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"* has a clear basis in the priority document.

3.1.5 Finally, the feature *"wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell"* has a basis at least in paragraphs [0093] and [00132] of **D7** (see pages 20 and 32).

3.1.6 In conclusion, the subject-matter of claim 1 of the Main Request is fully based on the disclosure of the priority document and entitled to the priority date of April 30, 2014. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 1.

3.2 Claim 2 of the Main Request is entitled to the priority date of April 30, 2004

The subject-matter of claim 2 of the Main Request has a basis in the priority document US 60/567,052 **D7**.

3.2.1 Basis for the features:

- *"An isolated polynucleotide that encodes a protein having alpha-ketoglutarate-dependent dioxygenase activity"*;
- *"wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 3 and SEQ ID NO: 4"*; and
- *"wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell"*

has already been provided in sections 3.1.1, 3.1.3, and 3.1.5 above.

3.2.2 A basis for the features *"wherein said protein enzymatically degrades (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins, (ii) an aryloxyphenoxypropionate herbicide, or (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* can be found at least in paragraphs [0019] and [0051] of D7 (see pages 6 and 9).

More specifically, paragraph [0019] of **D7** recites that

> *"This invention relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides."*

This paragraph provides a basis for the enzyme activities recited as alternatives (ii) and (iii) of claim 2. Further basis can be found in paragraph [0061] of **D7**.

Paragraph [0051] of **D7** teaches that

> *"An additional benefit of the AAD-1 gene is that unlike all tfdA homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (e.g., dichlorprop and mecoprop) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid)."*

This paragraph provides a basis for the enzyme activities recited in alternative (i) of claim 2.

3.2.3 In conclusion, the subject-matter of claim 2 of the Main Request is fully based on the disclosure in the priority document and entitled to the priority date of April 30, 2014. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 2.

3.3 Claim 4 of the Main Request is entitled to the priority date of April 30, 2004

Claim 4 of the Main Request is based on granted claim 3 but the definition of the enzymatic activity of the encoded protein has been amended. More specifically, the characterization of the protein as *"having R-specific alpha-ketoglutarate-dependent dioxygenase activity relative to a 2,4 D-herbicide"* has been amended to read *"having R-specific alpha-ketoglutarate-dependent dioxygenase activity relative to chiral phenoxy auxins"*.

A basis for this functional characterization of the enzymatic activity can be found in paragraph [0051] of the priority document (see page 9, lines 5-8 of **D7**; see also section 3.2.2 above).

In view of the above amendment, the Opponent's objection against the expression *"R-specific [...] activity relative to a 2,4 D-herbicide"* no longer applies.

In conclusion, the subject-matter of claim 4 of the Main Request is fully based on the disclosure in the priority document and is entitled to the priority date of April 30, 2014. Hence **D12** and D15 do not constitute prior art for the subject-matter of claim 4.

3.4 Claim 6 of the Main Request is entitled to the priority date of April 30, 2004

Claim 6 of the Main request is based on granted claim 5 and specifies that *"said promoter is a plant promoter or of viral origin, preferably a cassava vein mosaic virus promoter"*.

Literal bases for the terms *"plant promoter"* and *"promoter of viral origin"* can be found in paragraph [00140] of the priority document (see page 35 of **D7**).

A basis for the preferred *"cassava vein mosaic virus promoter"* can be found on page 49, second to last line, and on page 51, line 3. These two text passages refer to the CsMVM promoter. As will be understood by the skilled person and as explained in Table 14 of the opposed patent, the term "CsMVM promoter" is an abbreviation for "cassava vein mosaic virus promoter" (see legend at the bottom of Table 14 on page 66 of the application as originally filed).

Accordingly, the subject-matter of claim 6 of the Main Request is entitled to the priority date of April 30, 2014. Therefore, **D12** and **D15** do not constitute prior art for claim 6.

3.5 Claim 8 of the Main Request is entitled to the priority date of April 30, 2004

Claim 8 of the Main request is based on granted claim 7 and is directed to

> *"A method of detecting whether a plant comprises a polynucleotide of claim 1 or 2, wherein said method comprises collecting a sample from said plant and assaying said sample for the presence of said polynucleotide."*

The method of claim 8 is disclosed in paragraph [0019] of the priority document (see page 6, lines 11-14 of **D7**).

Thus, the subject-matter of claim 8 of the Main Request is based on the disclosure in the priority document and is entitled to the priority date of April 30, 2014. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 18.

3.6 Claim 10 of the Main Request is entitled to the priority date of April 30, 2004

Dependent claim 10 of the Main Request is based on granted claim 9 and further characterizes the claimed method as comprising a step of *"using a PCR primer or probe to detect for the presence of said polynucleotide"*.

The usage of PCT primers or probes for the identification of genes of the invention is disclosed in paragraph [0092] of the priority document (see page 19 of **D7**, lines 5 to 2 from the bottom).

Therefore, the subject-matter of claim 10 of the Main Request is based on the disclosure in the priority document and is entitled to the priority date of April 30, 2014. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 18.

3.7 Summarizing, at least the subject-matter of granted claims 1, 2, 4, 6, 8, and 10 of the Main Request is entitled to the priority date of April 30, 2004. Accordingly, **D12** and **D15** do not form prior art for the subject-matter of these claims.

4. Novelty; Articles 54 and 100(a) EPC

The Opponent raised novelty objections against the subject-matter of granted claims 1, 5, 7, 8 and 9, which correspond to claims 1, 2, 6, 8, 9, and 10 of the Main Request. The subject-matter of these claims was said to be anticipated by at least one of **D8**, **D9**, **D10**, **D11**, and **D12**.

These five novelty attacks are based on an identical line of argumentation, since all of **D8** to **D12** refer to genetically engineered plants comprising the *tfdA* gene from *Ralstonia eutropha* (previous name: *Alcaligenes eutrophus*; later renamed as: *Wautersia eutropha*; current name: *Cupriavidus necator*) operatively linked to the CaMV 35S promoter. The Opponent has submitted results of experiments as **D1** which allegedly demonstrate that the *tfdA* coding region hybridizes under "stringent conditions" with a nucleic acid having the nucleotide sequence of SEQ ID NO: 3. The Opponent concluded that the *tfdA* coding region would also hybridize with the complement of SEQ ID NO: 3. The Opponent further pointed out that the TfdA protein has α-ketoglutarate-dependent dioxygenase activity and that the CaMV35S promoter is functional in plant cells as required by granted claim 1.

In the enclosed Main Request, claim 1 was split into two independent claims. In amended claim 1, the encoded protein is further defined in structural terms. In amended claim 2, the encoded protein is further defined in functional terms.

4.1 The subject-matter of amended claim 1 is novel over the prior art

4.1.1 Without acquiescing with Opponent's assertion that the results of the experiments submitted as **D1** actually show that polynucleotides encoding TfdA hybridize **under stringent conditions with the full complement of a nucleic acid molecule having the nucleotide sequence of SEQ ID NO: 3** Patentee has decided to address this objection by amendment. Accordingly, amended claim 1 further characterizes the protein encoded by the claimed isolated polynucleotide in that it is

> *"at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"*

4.1.2 The Opponent has carried out a sequence alignment between the protein encoded by the *tfd*A gene of *Ralstonia eutropha* and the *aad1* gene of the Opposed Patent (see **D4**). According to this sequence alignment the protein encoded by the *tfd*A gene of *Ralstonia eutropha* exhibits about 32% amino acid sequence identity to the protein encoded by the *aad1* gene of the Opposed Patent. The polynucleotide sequence according to SEQ ID NO: 3 encodes the wild type AAD1 protein with the amino acid sequence according to SEQ ID NO: 9. The polynucleotide according to SEQ ID NO: 4 encodes the AAD1 protein according to SEQ ID NO: 10, which only differs from wt AAD1 in an Arg to Cys mutation at position 212 and a His to Ala mutation at position 2. The polynucleotide according to SEQ ID NO: 5 encodes the AAD1 protein according to SEQ ID NO: 11, which only differs from wt AAD1 protein by an additional Ala that is inserted between the N-terminal Met and the His at position 2. In its alignment Opponent appears to have used the wild type AAD1 amino acid sequence according to SEQ ID NO: 9 (it is noted that position 212 of the query sequence is Arg and not Cys and that position 15 is Ile which is only the case in the amino acid sequence according to SEQ ID NO: 9). Accordingly, the alignment with either the protein according to SEQ ID NO: 10 or 11 will lead to an even lower level of amino acid sequence identity than 32%.

4.1.3 Claim 1 of the Main Request requires that the polynucleotide encodes a protein that is at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, i.e. the proteins with the amino acid sequences according to SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11, respectively. As admitted by Opponent and further explained above, the protein encoded by the *tfdA* gene from *Ralstonia eutropha* exhibits 32% or less amino acid sequence identity to the amino acid sequences of SEQ ID NO: 9, 10 or 11. Therefore, the polynucleotides described in **D8, D9, D10, D11**, and **D12** do not anticipate the subject-matter of amended claim 1 and hence the subject-matter of claim 1 of the Main Request is novel over the prior art.

For the sake of completeness, it is noted again that **D12** does not even constitute prior art for the subject-matter of amended claim 1 of the Main Request (see section 3.1 above).

4.2 The subject-matter of amended claim 2 is novel over the prior art

4.2.1 Amended claim 2 characterizes the protein encoded by the isolated polynucleotide in that it

enzymatically degrades
(i) an R-enantiomer of chiral phenoxy auxin herbicide in addition to achiral phenoxy auxins,
(ii) an aryloxyphenoxypropionate herbicide, or
(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,

4.2.2 Documents **D8**, **D9**, **D10**, **D11**, and **D12** cited by the Opponent as relevant for novelty relate to transgenic plants comprising the *tfdA* gene from *Ralstonia eutropha*. The TdfA protein encoded by said gene is described as possessing achiral phenoxy auxin, i.e. 2,4-D, degrading activity. The TdfA protein cannot enzymatically degrade R-enantiomers of chiral phenoxy auxin herbicides as required by alternative (i) of amended claim 2. Further, the TdfA protein is not capable of degrading aryloxyphenoxypropionate herbicides as required by alternatives (ii) and (iii) of amended claim 2.

4.2.3 In summary: the protein encoded by the *tdf*A gene of *Ralstonia eutropha* is described as possessing achiral phenoxy auxin degrading activity in **D8**, **D9**, **D10**, **D11**, and **D12**. Moreover, the *tfdA* gene of *Ralstonia eutropha* does not possess the enzymatic activities recited in alternatives (i), (ii) and (iii) of amended claim 2. Therefore, the polynucleotides disclosed in **D8**, **D9**, **D10**, **D11**, and **D12** do not anticipate the subject-matter of amended claim 2. Hence the subject-matter of amended claim 2 is novel over the prior art.

For the sake of completeness, we would like to repeat that **D12** does not even constitute prior art for the subject-matter of amended claim 2 of the Main Request (see section 3.2 above).

4.3 The subject-matter of amended claims 3 to 11 is novel over the prior art

Amended claims 3 to 11 of the Main Request are directly or indirectly dependent on claim 1 or 2. Accordingly, the subject-matter of claims 3 to 11 derives its novelty from this dependency.

5. Inventive Step; Articles 56 and 100(a) EPC

5.1 The Opponent has alleged that claims 1, 5, 7, 8 and 9 as granted are anticipated by one or more of documents **D8** to **D12**, all of which are concerned with the known 2,4-D-herbicide degrading enzyme *tfda*. An objection of lack of inventive step is raised solely against the dependent claims 2 based upon **D8** (or any of **D9**, **D10**, **D11** and **D12**), when considered in combination with **D18**, against dependent claim 3 based upon **D8** (or any of **D9**, **D10**, **D11** and **D12**), when considered in combination with **D15**, against dependent claims 4 and 10 based upon **D8** (or any of **D9**, **D10**, **D11** and **D12**), when considered in combination with common general knowledge.

5.2 In this respect the Patentee reiterates that the claims of the Main Request can be considered entitled to the priority date of April 30, 2004 and thus **D12** and **D15** are not citable prior art.

Further, while not acquiescing to the Opponent's objection of lack of novelty over **D8** to **D11**, the independent claims of the Main Request presented herewith are amended to clearly recite additional features not disclosed in those documents.

For example, claim 1 now includes the feature that the encoded protein:

> *"is at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5".*

Claim 2 now includes the feature that the encoded protein enzymatically degrades

> *(i) An R-enantiomer of chiral phenoxy auxin herbicide in addition to achiral phenoxy auxins*
> *(ii) An aryloxyphenoxypropionate herbicide or*
> *(iii) A phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.*

It is submitted that claims 1 to 11 of the Main Request are both novel and inventive over all of the art cited by the Opponent and in the absence of any submissions to the contrary, the Patentee makes no further comments at this time. However, the Patentee reserves the right to make further submissions concerning the inventive step of the Main Request as necessary.

6. Sufficiency of Disclosure; Articles 83 and 100(b) EPC

6.1 The Opponent asserted that the invention as claimed in the granted claims is insufficiently disclosed, contrary to the provisions of Art. 83 EPC. The Opponent specifically attacked granted claims 1, 3 and 6.

The Opponent held the view that the specification provides support and disclosure only for polynucleotides having SEQ ID NOs: 3 to 5 but not for the broad definition based on stringent hybridization which allegedly covers polynucleotides encoding proteins having as little as 32% amino acid sequence identity with SEQ ID NO: 9. Allegedly, the broad scope of the granted claims is not congruent with the limited disclosure of the application.

6.2 The enclosed Main Request contains two independent claims that are each based on claim 1 as granted. Amended claim 1 is further limited in structural terms and amended claim 2 is further limited in functional terms (see sections 2.1 and 2.2 above). Claims 3 to 11 all directly or indirectly incorporate the limitations of independent claims 1 and 2 of the Main Request. In view of these limitations, the Opponent's above objection under Art. 83 EPC is moot.

6.3 The Opponent raised a further objection under Art. 83 against claim 3 as granted. This further objection is based on the fact that claim 3 characterizes the encoded protein as having R-specific alpha-ketoglutarate-dependent dioxygenase activity relative to a 2,4-D herbicide. The Opponent held the view that the specification of the Opposed Patent does not contain any disclosure for R-specific dioxygenases relative to 2,4-D.

In the enclosed Main Request, claim 3 as granted has been renumbered as claim 4 and has been amended by replacing the reference to 2,4-D by a reference to chiral phenoxy auxins. As already explained above (see section 2.4 above), this amendment is based on the disclosure in paragraph [0074] of the specification as original filed.

In view of this amendment, the Opponent's objection against claim 4 of the Main Request (corresponding to granted claim 3) is moot.

7. Summary

From the above discussion it is evident that the subject-matter claimed in the Main Request fulfils the requirements of Art. 54, 56, 83, 76(1), and 123(2) EPC.

Therefore, our request to maintain the Opposed Patent on the basis of the Main Request is fully justified.

Dr. Jörg Zwicker
European Patent Attorney

Enclosures:
Main Request (as marked-up copy and as clean copy)

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July 28, 2014
JZ/HE

Re.: **European Patent No. 2 308 977 B1 (Application No. 10 012 202.7 - 1401)**
Patent Proprietor: Dow AgroSciences LLC
Opposition by: Bayer CropScience NV
Our Ref.: 502-14 T4

This is in reply to the Opposition filed by Bayer CropScience NV of December 6, 2013 against EP 2 308 977 B1 and in response to the Communication of Notices of Opposition pursuant to Rule 79(1) EPC dated January 16, 2014.

1. Requests

1.1 It is requested that European Patent 2 308 977 B1 (in the following the: "Opposed Patent") be maintained on the basis of amended claims 1 to 22 of the enclosed Main Request.

1.2 Oral proceedings according to Art. 116(1) EPC are requested in the event that the Opposition Division cannot agree to the above Main Request.

2. Amendments; Articles 76(1), 123(2) and 100(c) EPC

As acknowledged by the Opponent the Opposed Patent was granted for a divisional application which was filed as an exact copy of the parent application as originally filed. The divisional application was not filed with claim listings, but rather the originally filed claims were copied as items into the description. Consequently, any subject-matter that has a basis

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under Art. 76(1) EPC in the parent application as originally filed (see PCT publication WO 2005/107437) will at the same time also have a basis under Art. 123(2) EPC in the text of the divisional application as originally filed and *vice versa*.

The Opponent asserted that the subject-matter of claims 1 and 19 contains subject-matter not disclosed in the parent application as filed, contrary to the provisions of Art. 76(1) EPC. Opponent's objections against the subject-matter of granted claims 1 and 19 primarily relied on the assertion that the definition of AAD-1 in the claims of the parent application was narrower than in the claims allowed in this divisional application. This is of course no basis for a valid objection under Art. 76(1) EPC since the parent and divisional applications as filed provide a broader definition of the gene and protein elsewhere in the application.

The subject-matter of the remaining claims (i.e. claims 2-18 and 20) was also attacked under Art. 76(1) EPC but only because they are dependent on claim 1 or claim 19. In addition, the Opponent asserted that all claims lacking a basis under Art. 76(1) EPC in the parent application would also lack a basis under Art. 123(2) EPC in the divisional application as filed.

The Patentee disagrees with these objections under Art. 76(1) and 123(2) EPC for the reasons outlined below. Since the Main Request filed herewith is based on the granted claims the objections raised by the Opponent will be discussed in as far as features objected to are also comprised in the claims of the Main Request.

2.1 Claim 1 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.1.1 The Opponent asserted that the text passages indicated in Patentee's letter to the EPO of January 27, 2011, namely claim 13 and paragraphs [00112], [00118], and [00124]-[00131] as well as paragraphs [0005] and [00117] of the parent application, would not provide a basis for the subject-matter of granted claim 1, contrary to the provisions of Art. 76(1) EPC. The Opponent particularly argued that originally filed claim 13 was limited to methods for controlling weeds in a field using plants expressing AAD-1 or a highly related variant thereof. The Opponent further argued that paragraphs [00112], [00118], and [00124]-[00131] of the parent application, cited in the response of January 27, 2011, describe hybridization conditions but do not make reference to a method of controlling weeds in a field or an area. The Opponent additionally asserted that the term *"area"* is used in paragraphs [0005] and [0007] of the parent application only in the context of glyphosate tolerant weeds and would not provide a basis to replace the term *"field"* by *"area"* in claim 1.

2.1.2 This analysis of the teaching of the parent application as originally filed is incorrect. Original claim 13 of the parent application (which refers to claim 5, which in turn refers to claim 1)

provides only one basis for the subject-matter of granted claim 1 and claim 1 of the Main Request. A very general disclosure for the method of claim 1 of the Main Request can be found in paragraphs [0020] and [0089] of the parent application as originally filed. Paragraph [0089] discloses that

> *"The subject invention also includes methods of controlling weeds wherein said methods comprise applying an AOPP herbicide and/or a phenoxy auxin herbicide to plants comprising an AAD-1 gene."* (Emphasis added)

A literal basis for the feature *"area"* can be found in paragraphs [0005], [0007] and [0017] of the parent application as originally filed. In particular, paragraph [0017] recites:

> *Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).* (Emphasis added)

The Opponent argued that the term *"area"* is used in paragraphs [0005] and [0007] only in connection with glyphosate use and glyphosate tolerant weeds (see page 9 of the Opposition Brief). The Opponent appears to be of the opinion that the feature *"area"* may not be separated from the feature *"glyphosate"*.

We disagree with the Opponent's position. In paragraph [0017] shown above, glyphosate is only mentioned by way of example (*"such as glyphosate"*). Thus, the methods of controlling weeds disclosed in the parent application are not limited to weeds resistant to glyphosate.

Summarizing, the feature *"A method of controlling at least one weed in an area"* finds a basis in paragraph [0020] or [0087] in combination with paragraph [0017] of the parent application as originally filed.

2.1.3 A basis for the feature *"wherein said area comprises at least one transgenic plant"* can be found in paragraph [0089] of the parent application as originally filed (see citation in section 2.1.2 above).

Paragraph [0089] discloses that an AOPP herbicide and/or a phenoxy auxin herbicide is applied *"to plants comprising an AAD-1 gene"*. Plants comprising the AAD-1 gene are necessarily transgenic plants. Accordingly, it is implicitly disclosed in paragraph [0089] that the area comprises at least one transgenic plant.

In addition, a literal basis for the term *"transgenic plant"* can be found in paragraph [00149] of the parent application as originally filed (see especially the second sentence of paragraph [00149]).

2.1.4 A basis for the feature *"said transgenic plant comprising an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* can be found at least in paragraphs [00149] and [0087] as originally filed in the parent application.

More specifically, paragraph [00149] discloses:

> *"The **protein-encoding genes of the subject invention** can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and **transgenic plants**."* (Emphasis added)

The protein-encoding genes of the subject invention are further explained in paragraph [0087] of the parent application:

> *"This invention also relates in part to the identification and use of genes encoding **aryloxyalkanoate dioxygenase enzymes** that are capable of degrading **phenoxy auxin and aryloxyphenoxypropionate herbicides**."* (Emphasis added)

Taken together, the feature *"said transgenic plant comprising an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* finds a direct and unambiguous basis in the parent application.

2.1.5 In comparison to claim 1 as granted, claim 1 of the Main Request has been amended to characterize the protein encoded by the isolated polynucleotide present in the transgenic plant in **structural** terms, namely in that said protein is *"at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"*.

A basis for this structural characterization can be found in paragraphs [0045], [0046], [0047], and [00120] of the parent application, which indicate SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5 to be polynucleotides encoding the most preferred proteins of the invention.

It is further taught in paragraph [0142] of the parent application

> *"Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it*

should be readily apparent that ***the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins****. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein."* (Emphasis added)

Thus, the parent application also discloses polynucleotides encoding proteins having amino acid similarity to a protein encoded by SEQ ID NO: 3, SEQ ID NO: 4 or SEQ ID NO: 5. The value of 70% amino acid sequence identity is explicitly recited in paragraph [0142] of the parent application.

Taken together, the feature "*wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5*" finds direct and unambiguous basis in the parent application.

2.1.6 The feature that "*said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9*" does not only find a basis in claim 5, which refers to claim 1 but also in the specification. For instance paragraph [0149] of the parent application:

"The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants."

and further in paragraph [0151] of the parent application:

"Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants."

Thus, paragraphs [0149] and [0151] of the parent application disclose that the transgenic plants of the invention can comprise **any** polynucleotide encoding a protein of the invention.

The polynucleotides encoding proteins of the invention are further specified in paragraph [0095] of the parent application:

"In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides in vitro." (Emphasis added)

It is set out in paragraph [0112] of the parent application:

"The subject invention uses classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides

that encode them is by defining a polynucleotide ***by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence*** *(the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences."* (Emphasis added)

Accordingly, the parent application does not only disclose transgenic plants comprising polynucleotides fulfilling the features of claim 1 of the parent application as alleged by the Opponent but also plants comprising nucleotide sequences that are characterized by their ability to hybridize with a nucleotide sequence encoding an exemplified protein. One particularly preferred protein is the native AAD-1 of *Sphingobium herbicidovorans* according to SEQ ID NO: 9 (see paragraph [0050] of the parent application).

Furthermore, it is taught in paragraph [0126] of the parent application:

"Thus, the subject invention includes polynucleotides (and/or their complements, ***preferably their full complements****) that hybridize with a polynucleotide exemplified herein. That is,* ***one way to define a gene*** *(and the protein it encodes), for example,* ***is by its ability to hybridize (under any of the conditions specifically disclosed herein)*** *with a known or specifically exemplified gene."* (Emphasis added)

Furthermore it is taught in paragraphs [0124] and [0127] of the parent application that "stringent conditions" are preferred hybridization conditions, when characterizing the polynucleotides encoding the proteins of the invention.

Taken together the feature *"said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO: 9"* finds basis in paragraphs [0050], [0095], [0112] [0124], [0126], [0127], [00149] and [00151] as well as in claims 1, 5 and 13 of the parent application.

2.1.7 A basis for the feature *"wherein said plant expresses said polynucleotide and produces said protein"* can be found in paragraph [00150] of the parent application as originally filed. In particular, paragraph [00150] discloses:

"In preferred embodiments, ***expression of the gene*** *results, directly or indirectly, in the intracellular* ***production*** *(and maintenance)* ***of the protein(s)*** *of interest. Plants can be rendered herbicide-resistant in this manner."* (Emphasis added)

Accordingly, the feature *"wherein said plant expresses said polynucleotide and produces said protein"* finds direct and unambiguous basis in the parent application.

2.1.8 A basis for the feature *"wherein said method comprises applying said herbicide to said area"* can be found in paragraph [0017] of the parent application as originally field (see citation of paragraph [0017] in section 2.1.2 above).

2.1.9 The parent application clearly provides direct and unambiguous basis for the subject-matter of claim 1 of the Main Request. Thus, the subject-matter of claim 1 of the Main Request fulfills the requirements of Art. 76(1) EPC.

As explained above, all paragraphs of the parent application as originally filed are identical to the paragraphs of the divisional application as originally filed and all claims of the parent application have been added on pages 159 to 168 of the divisional application. All subject-matter fulfilling the requirements of Art. 76(1) EPC as outlined in Sections 2.1.2 to 2.1.8, thus also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 1 is in accordance with the requirements of Articles 76(1) and 123(2) EPC.

2.2 Claim 2 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.2.1 Claim 2 of the Main Request is based on granted claim 1, which has been amended to further characterize the protein encoded by the isolated polynucleotide present in the transgenic plant in **functional** terms, namely by specifying the degrading activity of said protein. More specifically, the previous feature:

> *"enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"*

has been replaced by the expression

> *"enzymatically degrades*
> *(i) an R-enantiomer of chiral phenoxy auxin herbicide in addition to achiral phenoxy auxins,*
> *(ii) an aryloxyphenoxypropionate herbicide, or*
> *(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,"*

2.2.2 The feature *"a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* was recited in granted claim 1. Said feature has a basis in claim 13 originally filed in the parent application and also in paragraph [0087] of the parent application. As already acknowledged by the Opponent when concluding that the subject-matter of granted claim 1 lacked novelty (see aid feature encompasses the following three alternatives:

> *(1) "a phenoxy auxin herbicide"*
> *(2) "an aryloxyphenoxypropionate herbicide"*
> *(3) "a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"*

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In the enclosed amended claim 2, alternative (1) has been further specified in that the phenoxy auxin herbicide degrading activity is an activity that *"enzymatically degrades an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins"*. A literal basis for this feature can be found in paragraph [0074], fifth sentence, of the parent application. Alternative (2) and alternative (3) find basis in originally filed claim 47 and alternative (3) further in paragraph [0020], first sentence and paragraph [0087], first sentence.

All other features of granted claim 1, on which claim 2 is based, find basis in the parent application as filed as explained in sections 2.1.2, 2.1.3, 2.1.4, 2.1.6, 2.1.7, and 2.1.8 above and, thus, the subject-matter of claim 2 of the Main Request fulfils the requirements of Art. 76(1) EPC. All subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 2 of the Main Request is in accordance with the requirements of Art. 76(1) and 123(2) EPC.

2.3 Claim 20 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.3.1 The Opponent simply contended that the argumentation presented for claim 1 would apply *mutatis mutandis* to the subject-matter of granted claim 19, which corresponds to claim 20 of the Main Request.

We disagree with Opponent's above assertion. For example, the Opponent particularly argued that the usage of the term *"area"* in claim 1 contravenes the requirements of Art. 76 and Art. 123(2) EPC.

This term is not used in granted claim 19, which corresponds to claim 20 of the Main Request. Claim 20 of the Main Request is directed to a method of controlling weeds in a **field**. Already for this reason, the Opponent's line of argumentation does neither apply to granted claim 19 nor to claim 20 of the Main Request.

2.3.2 The feature *"Method of controlling at least one weed in a field"* finds a basis in original claim 13 of the parent application and in paragraphs [0020], [0078] and [0089] of the parent application as originally field, especially in the last sentence of paragraph [0078].

2.3.3 A basis for the feature *"wherein said field contains at least one plant that comprises an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an arylaxyphenoxypropionate"* can be found in original claim 13 of the parent application and in paragraphs [0078] and [0089] of the parent application as originally filed (see citation in section 2.1.2 above) in combination with paragraphs [0095] and [0020] of the parent application as originally filed.

Paragraph [0089] discloses that the method of controlling weeds comprises applying herbicides to *"to plants comprising an AAD-1 gene"*. As indicated above in section 2.3.2, paragraph [0078] provides a basis for a method of controlling a weed in a field. Thus, taken together, paragraphs [0078] and [0089] provide a basis for the feature that the field contains at least one plant comprising an AAD-1 gene.

It is further explained in paragraph [0095] that *"proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes"*. In in paragraph [0020] of the parent application it is stated that the enzymes of the subject invention (i.e. the AAD-1 enzymes) are capable of degrading phenoxy auxin and aryloxypheroxypropionate herbicides. Thus, a plant comprising an AAD-1 gene is *"a plant that comprises an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate"*, as required by claim 20 of the Main Request.

Summarizing the above, the feature *"wherein said field contains at least one plant that comprises an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate"* finds direct and unambiguous basis in the parent application as originally filed.

2.3.4 In comparison to claim 19 as granted, claim 20 of the Main Request has been amended to characterize the protein encoded by the isolated polynucleotide present in the transgenic plant in **structural** terms, namely in that said protein is *"at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"*.

A basis for this amendment can be found as outlined in detail in Section 2.1.5 above.

2.3.5 The feature *"wherein said nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence specified by SEQ ID NO:5"* finds a basis in the parent application, as set out in Section 2.1.6 above and further in paragraph [0047] of the parent application, i.e. in paragraphs [0047], [0095], [0112], [0120], [0124], [0126], [0127], [00149] and [00151] of the parent application.

2.3.6 The feature *"wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell"* finds a basis at least in paragraphs [00119] and [00158] of the parent application as filed.

2.3.7 The feature *"wherein the method comprises applying a **combination** of herbicides to said fields"* finds a basis in paragraph [0078] of the parent application; especially in the last sentence of paragraph [0078].

2.3.8 Summarizing, the parent application clearly provides direct and unambiguous basis for the subject-matter of claim 20 of the Main Request. Thus, the subject-matter of claim 20 of the Main Request fulfills the requirements of Art. 76(1) EPC.

As explained above, all subject-matter fulfilling the requirements of Art. 76(1) EPC as outlined in Sections 2.3.2 to 2.3.7 also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 20 of the Main Request is in accordance with the requirements of Articles 76(1) and 123(2) EPC.

2.4 Claims 21 of the Main Request fulfil the requirements of Art. 76(1) and Art. 123(2) EPC

2.4.1 Claim 21 of the Main Request is based on originally granted claim 19, which has been amended to further characterize the protein encoded by the isolated polynucleotide present in the transgenic plant in **functional** terms. Thus, this claim mirrors claim 2 discussed in Section 2.2 above. Accordingly, claim 21 finds basis as set out in Sections 2.3.2, 2.3.3, 2.3.5, 2.3.6, and 2.3.7 above and further regarding the functional feature as explained in Section 2.2. Accordingly, the subject-matter of claim 21 of the Main Request is in accordance with the requirements of Art. 76(1) and 123(2) EPC.

2.5 Claims 3-19 and 22 of the Main Request fulfil the requirements of Art. 76(1) and Art. 123(2) EPC

2.5.1 The Opponent contended that the subject-matter of granted claims 2-18 and 20, which correspond to claims 3-19 and 22, respectively, of the Main Request, contravenes the requirements of Art. 76(1) EPC. However, the objections against granted claims 2-18 and 20 were only based on the fact that said claims depend on claim 1 or claim 19.

2.5.2 Since the subject-matter of granted claims 1 and 19 as well as of claims 1, 2, 20 and 21 of the Main Request complies with the requirements of Art. 76(1) and Art. 123(2) EPC (see sections 2.1, 2.2, 2.3 and 2.4 above), the objections against dependent claims 3-19 and 22 of the Main Request are unfounded.

3. Entitlement to Priority, Art. 87 EPC

The Opponent held the view that the subject-matter of the granted claims is not entitled to the priority date of April 30, 2004 but only to the international filing date of May 2, 2005 and,

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therefore regarded **D12** (Bisht *et al.*, 2004) and **D15** (Schleinitz *et al.*, 2004) published after April 30, 2004 but prior to May 2, 2005 as prior art. He particularly asserted that the subject-matter of independent claims 1 and claim 19 does not have a basis in the first priority document filed on April 30, 2004 and that this also applied to the subject-matter of dependent claims 2-18 and 20 due to their reference to claim 1 and 19, respectively.

Granted claim 1 is split into two claims in the enclosed Main Request, namely into amended claims 1 and 2. In a similar manner, granted claim 19 is split into amended claims 20 and 21 in the enclosed Main Request. Thus, in the following it will be shown that claims 1, 2, 20 and 21 are entitled to the priority date of April 30, 2004.

3.1 Claim 1 of the Main Request is entitled to the priority date of April 30, 2004

The subject-matter of claim 1 of the Main Request has a basis in the priority document US 60/567,052 **D7**.

3.1.1 A general disclosure for the method of claim 1 of the Main Request can be found in paragraphs [0019] and [0063] of the priority document. Paragraph [0063] discloses that

> *"The subject invention also includes methods of controlling weeds wherein said methods comprise applying an AOPP herbicide and/or a phenoxy auxin herbicide to plants comprising an AAD-1 gene."* (Emphasis added)

A literal basis for the feature *"area"* can be found in paragraphs [0004], [0006] and [0016] of the priority document. In particular, paragraph [0016] recites:

> *Furthermore, the subject invention provides novel methods of preventing the development of, and controlling strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise (without the AAD-1 gene) be sensitive to that herbicide (such as 2,4-D).*

Summarizing, the feature *"A method of controlling at least one weed in an area"* finds a basis in paragraph [0019] or [0063] in combination with paragraph [0016] of the priority document.

3.1.2 A basis for the feature *"wherein said area comprises at least one transgenic plant"* can be found in paragraph [0063] of the priority document (see citation in section 3.1.1 above).

Paragraph [0063] discloses that an AOPP herbicide and/or a phenoxy auxin herbicide is applied to *"to plants comprising an AAD-1 gene"*. Plants comprising the AAD-1 gene are

necessarily transgenic plants. Accordingly, it is implicitly disclosed in paragraph [0063] that the area comprises at least one transgenic plant.

In addition, a literal basis for the term *"transgenic plant"* can be found in paragraph [00123] of the priority document (see especially the second sentence of paragraph [00123]).

3.1.3 A basis for the feature *"said transgenic plant comprising an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* can be found at least in paragraphs [00123] and [0061] of the priority document.

More specifically, paragraph [00123] discloses:

> *"The **protein-encoding genes of the subject invention** can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and **transgenic plants**."* (Emphasis added)

The protein-encoding genes of the subject invention are further explained in paragraph [0061] of the priority document:

> *"This invention also relates in part to the identification and use of genes encoding **aryloxyalkanoate dioxygenase enzymes** that are capable of degrading **phenoxy auxin and aryloxyphenoxypropionate herbicides**."* (Emphasis added)

Taken together, the feature *"said transgenic plant comprising an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* finds direct and unambiguous basis in the priority document.

3.1.4 A basis for the feature *"at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"* can be found at least in paragraphs [0035]-[0037], [0094], and [00116] of the priority document.

More specifically, paragraphs [0035], [0036], [0037] of the priority document indicate that SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5 are polynucleotides encoding the most preferred proteins of the invention.

It is further taught in paragraph [0116] of the priority document:

> *"Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it*

should be readily apparent that ***the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins****. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein."* (Emphasis added)

Thus, the parent application also discloses polynucleotides encoding proteins having amino acid similarity to a protein encoded by SEQ ID NO: 3, SEQ ID NO: 4 or SEQ ID NO: 5. The value of 70% amino acid sequence identity is explicitly recited in paragraph [00116] of the priority document (see page 28 of **D7**, line 2).

Taken together, the feature "*wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5*" finds direct and unambiguous basis in the priority document.

3.1.5 The feature that "*said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:5*" finds a basis in paragraph [00123] of the priority document:

"The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants."

and further in paragraph [00125] of the priority document:

"Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants."

Thus, paragraphs [00123] and [00125] of the priority document disclose that the transgenic plants of the invention can comprise **any** polynucleotide encoding a protein of the invention.

The polynucleotides encoding proteins of the invention are further specified in paragraph [0069] of the priority document:

"In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides in vitro." (Emphasis added)

It is set out in paragraph [0086] of the priority document:

"The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide ***by its ability to***

> ***hybridize, under a range of specified conditions, with an exemplified nucleotide sequence*** *(the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences."* (Emphasis added)

Accordingly, the priority document discloses plants comprising nucleotide sequences that are characterized by their ability to hybridize with a nucleotide sequence encoding an exemplified protein. One particularly preferred protein is the native AAD-1 of *Sphingobium herbicidovorans* according to SEQ ID NO: 9 (see paragraph [0040] of the priority document).

Furthermore, it is taught in paragraph [00100] of the priority document:

> *"Thus the subject invention includes polynucleotides (and/or their complements,* ***preferably their full complements****) that hybridize with a polynucleotide exemplified herein. That is,* ***one way to define a gene*** *(and the protein it encodes), for example,* ***is by its ability to hybridize (under any of the conditions specifically disclosed herein)*** *with a known or specifically exemplified gene."* (Emphasis added)

It is further taught in paragraphs [0098] and [00101] of the priority document that "stringent conditions" are preferred hybridization conditions, when characterizing the polynucleotides encoding the proteins of the invention.

Taken together the feature *"said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO: 9"* finds basis in paragraphs [0040], [0069], [0086], [0098], [00100], [00101], [00123] and [00125] of the priority document.

3.1.6 A basis for the feature *"wherein said plant expresses said polynucleotide and produces said protein"* can be found in paragraph [00124] of the priority document. In particular, paragraph [00124] discloses:

> *"In preferred embodiments,* ***expression of the gene*** *results, directly or indirectly, in the intracellular* ***production*** *(and maintenance)* ***of the protein(s)*** *of interest. Plants can be rendered herbicide-resistant in this manner."* (Emphasis added)

Accordingly, the feature *"wherein said plant expresses said polynucleotide and produces said protein"* finds direct and unambiguous basis in the priority document.

3.1.7 A basis for the feature *"wherein said method comprises applying said herbicide to said area"* can be found in paragraph [0016] of the priority document (see citation of paragraph [0016] in section 3.1.1 above).

3.1.8 In conclusion, the subject-matter of claim 1 of the Main Request is fully based on the disclosure of the priority document and entitled to the priority date of April 30, 2014. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 1.

3.2 Claim 2 of the Main Request is entitled to the priority date of April 30, 2004

The subject-matter of claim 2 of the Main Request has a basis in the priority document US 60/567,052 **D7**.

3.2.1 Basis for the features:

- *"A method of controlling at least one weed in an area";*
- *"wherein said area comprises at least one transgenic plant";*
- *"said transgenic plant comprising an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide";*
- *"said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9";*
- *"wherein said plant expresses said polynucleotide and produces said protein";*
- *"wherein said method comprises applying said herbicide to said area"*

has already been provided in sections 3.1.1 to 3.1.3 and 3.1.5 to 3.1.7 above.

3.2.2 A basis for the features *"that enzymatically degrades (i) an R-enantiomere of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins, (ii) an aryloxyphenoxypropionate herbicide, or (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* can be found at least in paragraphs [0019] and [0051] of **D7** (see pages 6 and 9).

More specifically, paragraph [0019] of **D7** recites that

> *"This invention relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides."*

This paragraph provides a basis for the enzyme activities recited as alternatives (ii) and (iii) of claim 2. Further basis can be found in paragraph [0061] of **D7**

Paragraph [0051] of **D7** teaches that

> *"An additional benefit of the AAD-1 gene is that unlike all tfdA homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (e.g., dichlorprop and mecoprop) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid)."*

This paragraph provides a basis for the enzyme activities recited in alternative (i) of claim 2.

3.2.3 In conclusion, the subject-matter of claim 2 of the Main Request is fully based on the disclosure in the priority document and entitled to the priority date of April 30, 2014. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 2.

3.3 Claim 20 of the Main Request is entitled to the priority date of April 30, 2004

The subject-matter of claim 20 of the Main Request has a basis in the priority document US 60/567,052 **D7.**

3.3.1 The feature *"Method of controlling at least one weed in a field"* finds a basis in paragraphs [0019], [0053] and [0063] of the priority document, especially in the last sentence of paragraph [0053].

3.3.2 A basis for the feature *"wherein said field contains at least one plant that comprises an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate"* can be found in paragraphs [0053] and [0063] of the priority document (see citation in section 3.1.1 above) in combination with paragraphs [0069] and [0019] of the priority document.

Paragraph [0063] discloses that the method of controlling weeds comprises applying herbicides to *"to plants comprising an AAD-1 gene"*. As indicated above in section 3.3.1, paragraph [0053] provides a basis for a method of controlling a weed in a field. Thus, taken together, paragraphs [0053] and [0063] provide a direct support for the feature that the field contains at least one plant comprising an AAD-1 gene.

It is further explained in paragraph [0069] that *"proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes"*. It is further explained in paragraph [0019] of the priority document that the enzymes of the subject invention (i.e. the AAD-1 enzymes) are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Thus, a plant comprising an AAD-1 gene is *"a plant that comprises an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate"*, as recited in amended claim 20 of the Main Request.

Summarizing the above, the feature *"wherein said field contains at least one plant that comprises an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate"* finds direct and unambiguous basis in the priority document.

3.3.3 A basis for the structural characterization *"at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"* can be found in paragraphs [0035], [0036], [0037], and [0094] of the priority document which indicate SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5 to be polynucleotides encoding the most preferred proteins of the invention.

It is further taught in paragraph [0142] of the priority document:

> *"Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that* ***the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins****. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein."* (Emphasis added)

Thus, the priority document also discloses polynucleotides encoding proteins having amino acid similarity to a protein encoded by SEQ ID NO: 3, SEQ ID NO: 4 or SEQ ID NO: 5. The value of 70% amino acid sequence identity is explicitly recited in paragraph [0116] of the priority document (see page 28, line 2 of **D7**).

Taken together, the feature *"wherein said protein is at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"* finds direct and unambiguous basis in the priority document.

3.3.4 The feature *"wherein said nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence specified by SEQ ID NO:5"* finds a basis in the priority document, as will be explained below:

Paragraph [00123] of the priority document discloses:

> *"The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants."*

and further in paragraph [00125] of the priority document:

> *"Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants."*

Thus, paragraphs [00123] and [00125] of the priority document disclose that the transgenic plants of the invention can comprise **any** polynucleotide encoding a protein of the invention.

The polynucleotides encoding proteins of the invention are further specified in paragraph [0069] of the priority document:

> *"In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides in vitro."* (Emphasis added)

It is set out in paragraph [0086] of the priority document:

> *"The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide* ***by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence*** *(the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences."* (Emphasis added)

Accordingly, the priority document discloses plants comprising nucleotide sequences that are characterized by their ability to hybridize with a nucleotide sequence encoding an exemplified protein. One particularly preferred nucleotide sequence is the "plant-optimized" DNA sequence *AAD-1 (v3)* according to SEQ ID NO: 5 (see paragraph [0037] of the priority document).

The Opponent particularly asserted that SEQ ID NO: 5 disclosed in the priority document is not identical to SEQ ID NO: 5 disclosed in the Opposed Patent as originally filed. Allegedly, the 3' ends would differ between the two sequences. We disagree with this allegation. The last five pages of the priority document contain sequence information. SEQ ID NO: 5 is shown on the second page of these five pages. The opposed patent is a divisional application, and the sequence listing for the present divisional application was filed with the EPO on September 30, 2010. This originally filed sequence listing contains nineteen pages. SEQ ID NO: 5 is shown on page 3 of the sequence listing and is identical to SEQ ID NO: 5 of the priority document. In particular, contrary to the Opponent's assertions, both sequences have the same 3' end, namely GCTTAATCAC CTAGAGCTC.

Further, it is taught in paragraph [0100] of the priority document:

> *"Thus, the subject invention includes polynucleotides (and/or their complements,* ***preferably their full complements****) that hybridize with a polynucleotide exemplified herein. That is,* ***one way to define a gene*** *(and the protein it encodes), for example,* ***is by its ability to hybridize (under any of the conditions specifically disclosed herein)*** *with a known or specifically exemplified gene."* (Emphasis added)

Furthermore it is taught in paragraphs [0098] and [00101] of the priority document that "stringent conditions" are preferred hybridization conditions, when characterizing the polynucleotides encoding the proteins of the invention.

Taken together the feature *"wherein said nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence specified by SEQ ID NO: 5"* finds basis in paragraphs [0037], [0069], [0086], [0098], [00100], [00101], [00123] and [00125] of the priority document.

3.3.5 The feature *"wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell"* finds a basis at least in paragraphs [0093] and [00132] of the priority document

3.3.6 The feature *"wherein the method comprises applying a **combination** of herbicides to said fields"* finds a basis in paragraph [0053] of the priority document; especially in the last sentence of paragraph [0053].

3.3.7 In conclusion, the subject-matter of claim 20 of the Main Request is fully based on the disclosure in the priority document and entitled to the priority date of April 30, 2014. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 20.

3.4 Claim 21 of the Main Request is entitled to the priority date of April 30, 2004

The subject-matter of claim 21 of the Main Request has a basis in the priority document US 60/567,052 **D7**.

3.4.1 Basis for the features:

- *"Method of controlling at least one weed in a field";*
- *"wherein said field contains at least one plant that comprises an isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate";*
- *"wherein said nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence specified by SEQ ID NO:5";*
- *"wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell";* and
- *"wherein the method comprises applying a combination of herbicides to said fields"*

has already been provided in sections 3.3.1 to 3.3.2 and 3.3.4 to 3.3.6 above.

3.4.2 A basis for the features *"that enzymatically degrades (i) an R-enantiomere of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins, (ii) an aryloxyphenoxypropionate*

herbicide, or (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide" can be found at least in paragraphs [0019] and [0051] of **D7** (see pages 6 and 9).

More specifically, paragraph [0019] of **D7** recites that

> *"This invention relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides."*

This paragraph provides a basis for the enzyme activities recited as alternatives (ii) and (iii) of claim 21.

Paragraph [0051] of **D7** teaches that

> *"An additional benefit of the AAD-1 gene is that unlike all tfdA homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (e.g., dichlorprop and mecoprop) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid)."*

This paragraph provides a basis for the enzyme activities recited in alternative (i) of claim 21.

3.4.3 In conclusion, the subject-matter of claim 21 of the Main Request is fully based on the disclosure in the priority document and entitled to the priority date of April 30, 2014. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 21.

3.5 Summarizing, the subject-matter of independent claims 1, 2, 20 and 21 of the Main Request is entitled to the priority date of April 30, 2004.

The Opponent asserted that the subject-matter of the dependent claims does not validly claim the priority date of April 30, 2004, because they are dependent on granted claims 1 and 19 that are not entitled to this priority date. However, as shown in sections 3.1 to 3.4, all independent claims validly claim the priority date of April 30, 2004. The Opponent's assertion that the dependent claims do not validly claim the priority date is therefore unfounded.

Accordingly, **D12** and **D15** do not form prior art for the subject-matter of any claim in the enclosed Main Request.

4. Novelty; Articles 54 and 100(a) EPC

The Opponent raised novelty objections against the subject-matter of granted claims 1-3, 11-12 and 18, which correspond to claims 1-4, 12-13 and 19 of the Main Request. The subject-matter of these claims was said to be anticipated by one or more of **D8**, **D9**, D10, D11, and **D12**.

These five novelty attacks are based on an identical line of argumentation, since all of **D8** to **D12** refer to genetically engineered plants comprising the *tfdA* gene from *Ralstonia eutropha* (previous name: *Alcaligenes eutrophus*; later renamed as: *Wautersia eutropha*; current name: *Cupriavidus necator*). The transgenic plants were sprayed with the herbicide 2,4-D. The Opponent has submitted results of experiments as **D1** which allegedly demonstrate that the *tfdA* gene hybridizes under "stringent conditions" with a nucleic acid encoding a protein of SEQ ID NO: 9 as recited in claim 1. The Opponent further pointed out that the TfdA protein degrades 2,4-D (i.e. a phenoxy auxin herbicide) as required in one alternative by granted claim 1.

In the enclosed Main Request claim 1 was split into two independent claims. In amended claim 1, the encoded protein is further defined in structural terms. In amended claim 2, the encoded protein is further defined in functional terms.

4.1 The subject-matter of amended claim 1 is novel over the prior art

4.1.1 Without acquiescing with Opponents assertion that the results of the experiments submitted as **D1** actually show that polynucleotides encoding TfdA hybridizes **under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9** Patentee has decided to address this objection by amendment. Accordingly, amended claim 1 further characterizes the protein encoded by the isolated polynucleotide present within the transgenic plant in that it is

> *"at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"*

4.1.2 The Opponent has carried out a sequence alignment between the protein encoded by the *tfd*A gene of *Ralstonia eutropha* and the *aad1* gene of the Opposed Patent (see **D4**). According to this sequence alignment the protein encoded by the *tfd*A gene of *Ralstonia eutropha* exhibits about 32% amino acid sequence identity to the protein encoded by the *aad1* gene of the Opposed Patent. The polynucleotide sequence according to SEQ ID NO: 3 encodes the wild type AAD1 protein with the amino acid sequence according to SEQ ID NO: 9. The polynucleotide according to SEQ ID NO: 4 encodes the AAD1 protein according to SEQ ID NO: 10, which only differs from wt AAD1 in an Arg to Cys mutation at position 212 and a

His to Ala mutation at position 2. The polynucleotide according to SEQ ID NO: 5 encodes the AAD1 protein according to SEQ ID NO: 11, which only differs from wt AAD1 protein by an additional Ala that is inserted between the N-terminal Met and the His at position 2. In its alignment Opponent appears to have used the wild type AAD1 amino acid sequence according to SEQ ID NO: 9 (it is noted that position 212 of the query sequence is Arg and not Cys and that position 15 is Ile which is only the case in the amino acid sequence according to SEQ ID NO: 9). Accordingly, the alignment with either the protein according to SEQ ID NO: 10 or 11 will lead to an even lower level of amino acid sequence identity than 32%.

4.1.3 Claim 1 of the Main Request requires that the transgenic plant comprises an isolated polynucleotide encoding a protein that is at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, i.e. the proteins with the amino acid sequences according to SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11, respectively. As admitted by Opponent and further explained above, the protein encoded by the *tfdA* gene from *Ralstonia eutropha* exhibits 32% or less amino acid sequence identity to the amino acid sequences of SEQ ID NO: 9, 10 or 11. Therefore, the transgenic plants described in **D8**, **D9**, **D10**, **D11**, and **D12** as well as the methods using such transgenic plants do not anticipate the subject-matter of amended claim 1. Hence the subject-matter of claim 1 of the Main Request is novel over the prior art.

For the sake of completeness, it is noted again that **D12** does not even constitute prior art for the subject-matter of amended claim 1 of the Main Request (see section 3.1.8 above).

4.2 The subject-matter of amended claim 2 is novel over the prior art

4.2.1 Amended claim 2 characterizes the protein encoded by the isolated polynucleotide present within the transgenic plant in that it

enzymatically degrades
(i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,
(ii) an aryloxyphenoxypropionate herbicide, or
(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,

4.2.2 Documents **D8**, **D9**, **D10**, **D11**, and **D12** cited by the Opponent as relevant for novelty describe methods using transgenic plants comprising the *tfdA* gene from *Ralstonia eutropha*. The TdfA protein encoded by said gene is described as possessing achiral phenoxy auxin, i.e. 2,4-D, degrading activity. The TdfA protein cannot enzymatically degrade R-enantiomers of chiral phenoxy auxin herbicides as required by alternative (i) of amended claim 2. Further, the TdfA protein is not capable of degrading aryloxyphenoxypropionate herbicides as required by alternatives (ii) and (iii) of amended claim 2.

4.2.3 In summary: the protein encoded by the *tfdA* gene of *Ralstonia eutropha* is described as possessing achiral phenoxy auxin degrading activity in **D8**, **D9**, **D10**, **D11**, and **D12**. Moreover, the *tfdA* gene of *Ralstonia eutropha* does not possess the enzymatic activities recited in alternatives (i), (ii) and (iii) of amended claim 2. Therefore, the transgenic plants disclosed in **D8**, **D9**, **D10**, **D11**, and **D12** as well as the methods using such transgenic plants do not anticipate the subject-matter of amended claim 2. Hence the subject-matter of amended claim 2 is novel over the prior art.

For the sake of completeness, we would like to repeat that **D12** does not even constitute prior art for the subject-matter of amended claim 2 of the Main Request (see section 3.2.3 above).

4.3 The subject-matter of amended claim 20 is novel over the prior art

4.3.1 Amended claim 20 is based on granted claim 19 and has been amended by characterizing the protein encoded by the isolated polynucleotide present within the transgenic plant in that said protein is

> *"at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"*

In other words, the limitation introduced into amended claim 20 is the same limitation that has been introduced into amended claim 1.

4.3.2 As explained above in sections 4.1.2 and 4.1.3, the *tfdA* gene of *Ralstonia eutropha* present in transgenic plants of **D8**, **D9**, **D10**, **D11**, and **D12** encodes a protein that exhibits only 32% sequence identity to a protein encoded by SEQ ID NO: 3, 4 or 5. Accordingly, the transgenic plants described in **D8**, **D9**, **D10**, **D11**, and **D12** and the methods using such transgenic plants cannot anticipate the method of amended claimed 20. Hence the subject-matter of claim 20 of the Main Request is novel over the prior art.

For the sake of completeness, it is noted again that **D12** does not even constitute prior art for the subject-matter of amended claim 20 of the Main Request (see section 3.3.7 above).

4.4 The subject-matter of amended claim 21 is novel over the prior art

4.4.1 Amended claim 21 is based on granted claim 19 and has been amended by characterizing the protein encoded by the isolated polynucleotide present within the transgenic plant in that said protein

> *enzymatically degrades*
> *(i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins,*
> *(ii) an aryloxyphenoxypropionate herbicide, or*

(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,

In other words, the limitations introduced into amended claim 21 are the same limitations that have been introduced into amended claim 2.

4.4.2 As explained above in sections 4.2.2 and 4.2.3, Documents **D8**, **D9**, **D10**, **D11**, and **D12** describe methods using transgenic plants comprising the *tfdA* gene from *Ralstonia eutropha*. The protein encoded by the *tfdA* gene of *Ralstonia eutropha* is only capable of degrading achiral phenoxy auxins but lacks the enzymatic activities recited in alternatives (i), (ii) and (iii) of amended claim 21. Therefore, the transgenic plants disclosed in **D8**, **D9**, **D10**, **D11**, and **D12** as well as the methods using such transgenic plants do not anticipate the subject-matter of amended claim 21. Hence the subject-matter of amended claim 21 is novel over the prior art.

For the sake of completeness, it is noted again that **D12** does not even constitute prior art for the subject-matter of amended claim 21 of the Main Request (see section 3.4.3 above).

4.5 <u>The subject-matter of amended claims 3 to 19 and 22 is novel over the prior art</u>

Amended claims 3 to 19 of the Main Request are directly or indirectly dependent on claim 1 or 2. Amended claim 22 of the Main Request is directly dependent on claims 20 and 21. Accordingly, the subject-matter of claims 3 to 19 and 22 derives its novelty from these dependencies.

5. <u>Inventive Step; Articles 56 and 100(a) EPC</u>

5.1 The Opponent has alleged that claims 1 to 3, 11, 12 and 18 as granted are anticipated by one or more of documents **D8** to **D12**, all of which are concerned with the known 2,4-D-herbicide degrading enzyme *tfdA*. An objection of lack of inventive step is raised solely against the dependent claims 5 to 9 based upon **D8** (or any of **D9**, **D10**, **D11** and **D12**), when considered in combination with **D19**, **D20**, **D21**, or **D22**, against dependent claim 13 based upon **D8** (or any of **D9**, **D10**, **D11** and **D12**), apparently when considered in combination with common general knowledge (it is noted that the Opponent does not specifically mention in Section 7.1.2 of the Opposition brief with which disclosure the closest prior art is combined), and against dependent claim 14 based upon **D8** (or any of **D9**, **D10**, **D11** and **D12**), when considered in combination with **D15**.

5.2 In this respect the Patentee reiterates that the claims of the Main Request can be considered entitled to the priority date of April 30, 2004 and thus **D12** and **D15** are not citable prior art.

Further, while not acquiescing to the Opponent's objection of lack of novelty over **D8** to **D11**, the independent claims of the Main Request presented herewith are amended to clearly recite additional features not disclosed in those documents.

For example, claim 1 and claim 20 now include the feature that the encoded protein:

> *"is at least 70% identical with a protein encoded by a sequence selected from the group consisting of SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5".*

Claim 2 and claim 21 now include the feature that the encoded protein enzymatically degrades

> *(i) An R-enantiomer of chiral phenoxy auxin herbicide in addition to achiral phenoxy auxins*
> *(ii) An aryloxyphenoxypropionate herbicide or*
> *(iii) A phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.*

It is submitted that claims 1 to 22 of the Main Request are both novel and inventive over all of the art cited by the Opponent and in the absence of any submissions to the contrary, the Patentee makes no further comments at this time. However, the Patentee reserves the right to make further submissions concerning the inventive step of the Main Request as necessary.

6. Sufficiency of Disclosure; Articles 83 and 100(b) EPC

6.1 The Opponent asserted that the invention as claimed in the granted claims is insufficiently disclosed, contrary to the provisions of Art. 83 EPC. The Opponent held the view that the specification provides support and disclosure only for methods using polynucleotides having SEQ ID NOs: 3 to 5 but not for the broad definition based on stringent hybridization which allegedly covers polynucleotides encoding proteins having as little as 32% amino acid sequence identity with SEQ ID NO: 9. Allegedly, the broad scope of the granted claims is not congruent with the limited disclosure of the application.

6.2 The enclosed Main Request contains four independent claims that are based on claim 1 as granted or on claim 19 as granted. Amended claims 1 and 20 are further limited in structural terms and amended claims 2 and 21 are further limited in functional terms (see sections 2.1, 2.2, 2.3 and 2.4 above). Claims 3 to 19 all directly or indirectly incorporate the limitations of independent claims 1 and 2 of the Main Request. Claim 22 directly incorporates the limitations of independent claims 20 or 21 of the Main Request. In view of these limitations, the Opponent's above objection under Art. 83 EPC is moot.

6.3 The Opponent additionally raised a specific objection against granted claim 17. This objection is discussed in section 8 of the Opposition Brief, which is entitled *"Sufficiency of Disclosure (Articles 83 EPC and 100(b) EPC)"*. However, the Opponent asserts that *"It is **unclear** [...] how the term "parent herbicide" connects with the method of controlling weeds in an area"* (see page 18 of the Opposition Brief; emphasis added). Thus, Opponent's objection against claim 17 is a clarity objection under Art. 84 EPC.

According to Art. 100 EPC, an opposition may **only** be filed on the grounds recited in paragraphs (a), (b) and (c) of Art. 100 EPC. In other words, the list of grounds for opposition in Art. 100 EPC is exhaustive. Lack of clarity is not listed as a ground for opposition and can, thus, not be a basis to revoke this patent. Therefore, the Patentee sees no reason to further discuss the Opponent's objection against claim 17.

7. Double Patenting

7.1 In a remark on page 18 of the Opposition Brief, the Opponent alleged that granted claim 14, which corresponds to claim 15 of the Main Request claims the same subject-matter as claim 16 granted in the parent patent EP 1 740 039 B1.

7.2 According to Art. 100 EPC, an opposition may **only** be filed on the grounds recited in paragraphs (a), (b) and (c) of Art. 100 EPC. Double patenting is not listed as a ground for opposition and can, thus, not be a basis to revoke this patent (see, e.g. **T 936/04**). Consequently, Patentee sees no reason to further address Opponent's allegation.

8. Summary

From the above discussion it is evident that the subject-matter claimed in the Main Request fulfils the requirements of Art. 54, 56, 83, 76(1), and 123(2) EPC.

Therefore, our request to maintain the Opposed Patent on the basis of the Main Request is fully justified.

Dr. Mark Zwicker
European Patent Attorney

Enclosures:
Main Request (marked-up copy and as clean copy)

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November 24, 2014
JZ/HE

Re.: European Patent No. 2 319 932 B1 (Application No. 10 012 196.5 - 1401)
Patent Proprietor: Dow AgroSciences LLC
Opposition by: Bayer CropScience NV
Our Ref.: 802-14 T1

This is in reply to the Opposition filed by Bayer CropScience NV on June 18, 2014 against EP 2 319 932 B1 and in response to the Communication of Notices of Opposition pursuant to Rule 79(1) EPC dated July 23, 2014.

1. Requests

1.1 It is requested that European Patent 2 319 932 B1 (in the following: the Opposed Patent) be maintained on the basis of amended claims 1 to 17 of the enclosed Main Request.

1.2 Oral proceedings according to Art. 116(1) EPC are requested in the event that the Opposition Division cannot agree to the above Main Request.

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2. Amendments: Articles 76(1), 123(2) and 100(c) EPC

As acknowledged by the Opponent, the Opposed Patent was granted for a divisional application which was filed as an exact copy of the parent application as originally filed. The divisional application was not filed with a claim listing, but rather the originally filed claims were copied as items into the description. Consequently, any subject-matter that has a basis under Art. 76(1) EPC in the parent application as originally filed (see PCT publication WO 2005/107437) will at the same time also have a basis under Art. 123(2) EPC in the text of the divisional application as originally filed and *vice versa*.

The Opponent asserted that the subject-matter of claims 1, 6 and 15 contains subject-matter not disclosed in the parent application as filed, contrary to the provisions of Art. 76(1) EPC. Opponent's objections against the subject-matter of granted claims 1, 6 and 15 primarily relied on the assertion that the definition of AAD-1 in the claims of the parent application was narrower than in the claims allowed in this divisional application. This is of course no basis for a valid objection under Art. 76(1) EPC, since the parent and divisional applications as filed provide a broader definition of the gene and protein elsewhere in the application.

The subject-matter of the remaining claims (i.e. claims 2-5, 7-14 and 16) was also attacked under Art. 76(1) EPC, merely due to their dependency on claim 1. In addition, the Opponent asserted that all claims lacking a basis under Art. 76(1) EPC in the parent application would also lack a basis under Art. 123(2) EPC in the divisional application as filed.

The Patentee disagrees with these objections under Art. 76(1) and 123(2) EPC for the reasons outlined below. Since the Main Request filed herewith is based on the originally granted claims the objections raised by the opponents will be discussed in as far as features objected to are also comprised in the claims of the Main Request.

2.1 Claim 1 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.1.1 The Opponent asserted that the text passages indicated in Patentee's letter to the EPO of January 28, 2011, namely originally filed claims 1, 6 and 8, and paragraphs [00126]-[00137] of the parent application, would not provide a basis for the subject-matter of granted claim 1, contrary to the provisions of Art. 76 EPC.

The Opponent argued in particular that originally filed claim 1 "*was limited to plants expressing AAD-1 or a highly related variant thereof having a particular enzymatic activity*" (i.e., aryloxyalkanoate dioxygenase activity), while the transgenic plant cell of granted claim 1 extends to "*transgenic plant cells comprising any polynucleotide hybridizing* "to any nucleotide molecule that encodes AAD-1" *of any enzymatic activity which may degrade phenoxy auxin*

herbicides or aryloxyphenoxypropionate herbicide" (see page 8, second paragraph of the Opposition brief).

The Opponent further argued that paragraphs [00126]-[00127] of the parent application, cited in the response of January 28, 2011, do not make reference to the specific combination of characteristics now claimed.

2.1.2 This analysis of the teaching of the parent application as originally filed is incorrect. Claim 1 of the parent application characterizes the variant as having *"arylalkanoate dioxygenase activity"*. The enzymatic activity is further described in paragraph [0020] of the parent application:

> *"This invention also relates in part to the identification and use of genes encoding* ***aryloxyalkanoate dioxygenase enzymes*** *that are capable of degrading* ***phenoxy auxin and aryloxyphenoxypropionate herbicides****."* (Emphasis added)

This statement is also repeated in the first sentence of paragraph [0087] of the parent application.

Thus, the feature that the *"protein enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* is based on claim 1 of the parent application and the more precise definition of the enzymatic activity in paragraphs [0020] and [0087] of the parent application.

2.1.3 The feature that *"said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9"* finds a basis in the specification. For instance, paragraph [0149] of the parent application supports that said polynucleotide is comprised in transgenic plant cells:

> *"The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants."* (Emphasis added)

Thus, paragraph [0149] of the parent application discloses that the transgenic plant cells of the invention can comprise **any** polynucleotide encoding a protein of the invention.

The polynucleotides encoding proteins of the invention are further specified in paragraph [0095] of the parent application:

> *"In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides in vitro."*

It should particular be noted that the term *"genes and proteins of the invention"* does not only relate to the specifically disclosed polynucleotide and polypeptide sequences, as explained in paragraph [00135] of the parent application:

> *The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity.*

It is further set out in paragraph [0112] of the parent application:

> *"The subject invention uses classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide* ***by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence*** *(the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences."* (Emphasis added)

Accordingly, the parent application does not only disclose transgenic plant cells comprising polynucleotides fulfilling the features of claim 1 of the parent application as alleged by the Opponent but also plant cells comprising nucleotide sequences that are characterized by their ability to hybridize with a nucleotide sequence encoding an exemplified protein. One particularly preferred protein is the native AAD-1 of *Sphingobium herbicidovorans* according to SEQ ID NO: 9 (see paragraph [0050] of the parent application).

Furthermore, it is taught in paragraph [0126] of the parent application:

> *"Thus, the subject invention includes polynucleotides (and/or their complements,* ***preferably their full complements****) that hybridize with a polynucleotide exemplified herein. That is,* ***one way to define a gene*** *(and the protein it encodes), for example,* ***is by its ability to hybridize (under any of the conditions specifically disclosed herein)*** *with a known or specifically exemplified gene"* (Emphasis added)

Furthermore it is taught in paragraphs [0124] and [0127] of the parent application that *"stringent conditions"* are preferred hybridization conditions, when characterizing the polynucleotides encoding the proteins of the invention.

Taken together the feature *"said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9"* finds basis in

paragraphs [0050], [0095], [0112], [0124], [0126], [0127], [00135], and [00149] as well as in claim 1 of the parent application.

2.1.4 The feature that "*said plant expresses said polynucleotide and produces said protein*" finds a basis in paragraph [00150] of the parent application as filed (see pages 36-37). Said paragraph is part of a section discussing transgenic hosts, including transgenic plant hosts and transgenic plant cells. More specifically, the first three sentences of paragraph [00150] read:

> *In preferred embodiments, expression of the gene results, directly or indirectly in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells.* (Emphasis added)

Accordingly, the feature "*said plant expresses said polynucleotide and produces said protein*" finds direct and unambiguous basis in the parent application.

2.1.5 Claim 1 of the Main Request has been further amended to characterize the protein encoded by the isolated polynucleotide present in the transgenic plant in **structural** terms, namely in that said protein is *"at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"*.

A basis for this structural characterization can be found in paragraphs [0045], [0046], [0047], and [0120] of the parent application which indicate SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5 to be polynucleotides encoding the most preferred proteins of the invention.

It is further taught in paragraph [0142] of the parent application

> *"Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that **the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins**. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein."* (Emphasis added)

Thus, the parent application also discloses polynucleotides encoding proteins having amino acid similarity to a protein encoded by SEQ ID NO: 3, SEQ ID NO: 4 or SEQ ID NO: 5. The value of 70% amino acid sequence identity is explicitly recited in paragraph [0142] of the parent application.

Taken together the feature "*wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5*" finds direct and unambiguous basis in the parent application.

2.1.6 The parent application clearly provides direct and unambiguous basis for the subject-matter of claim 1 of the Main Request. Thus, the subject-matter of claim 1 of the Main Request fulfills the requirements of Art. 76(1) EPC.

As explained above all paragraphs of the parent application as originally filed are identical to the paragraphs of the divisional application as originally filed and all claims of the parent application have been added on pages 159 to 168 of the divisional application. All subject-matter fulfilling the requirements of Art. 76(1) EPC as outlined in Sections 2.1.1 to 2.1.5, thus also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 1 is in accordance with the requirements of Articles 76(1) and 123(2) EPC.

2.2 Claim 2 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.2.1 Claim 2 of the Main Request is based on granted claim 1, which has been amended to further characterize the protein encoded by the isolated polynucleotide present in the transgenic plant cells in **functional** terms, namely by specifying the degrading activity of said protein. More specifically, the previous expression

"enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"

has been replaced by the following expression:

"enzymatically degrades
(i) an R-enantiomer of chiral phenoxy auxin herbicide in addition to achiral phenoxy auxins,
(ii) an aryloxyphenoxypropionate herbicide, or
(iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide,"

2.2.2 The Opponent already acknowledged through the interpretation of the previous feature *"enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* (see page 6, paragraph 3 of the Opposition brief) that the feature of granted claim 1 encompasses the following three alternatives:

(1) "a phenoxy auxin herbicide"
(2) "an aryloxyphenoxypropionate herbicide"
(3) "a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"

Alternative (1) has been further specified in that the phenoxy auxin herbicide degrading activity is an activity that "*enzymatically degrades an R-enantiomer of chiral phenoxy auxin herbicides*

in addition to achiral phenoxy auxins". A literal basis for this feature can be found in paragraph [0074], fifth sentence, of the parent application.

All other features of granted claim 1, on which claim 2 is based, find basis in the parent application as filed as explained in sections 2.1.2 and 2.1.4 above. Thus, the subject-matter of claim 2 of the Main Request fulfils the requirements of Art. 76(1) EPC. All subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 2 of the Main Request is in accordance with the requirements of Art. 76(1) and 123(2) EPC.

2.3 Claim 7 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.3.1 The Opponent asserted that granted claim 6, which corresponds to claim 7 of the Main Request does not fulfil the requirements of Articles 76(1) and 123(2) EPC. The Opponent pointed out in particular that granted claim 6 defines the plant cell as comprising *"a glyphosate tolerance trait and a glufosinate tolerance trait"*. The Opponent argued that paragraphs [0016], [0082], [0098], and [00344] as well as Tables 25 and 346 of the application as filed do not provide an adequate basis for these two tolerance traits. Allegedly, the recitation of only two tolerance traits, i.e. *"a glyphosate tolerance trait and a glufosinate tolerance trait"*, constitutes the singling-out of a specific combination of tolerance traits.

2.3.2 The Opponent's argument is flawed. Paragraph [0016] of the parent application teaches that:

> *"The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate, imidazolinone-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options."* (Emphasis added)

Paragraph [0016] of the parent application, thus provides a list of three herbicide resistance genes, namely, glyphosate, imidazolinone-, and glufosinate-resistance genes. One of the preferred embodiments is deleted from the list of three elements. The deletion of one preferred embodiment from a list of three preferred embodiments is in agreement with the requirements of Art. 76(1) EPC in line with the principles set out in Enlarged Board of Appeal Decision **G 2/10** regarding the permissibility of disclaiming an embodiment that is taught to belong to the invention under Art. 123(2) EPC. The deletion (disclaiming) of subject-matter taught as forming part of the invention is permissible, if the subject-matter remaining in the claim after the deletion of this element is directly and unambiguously disclosed (see Headnote 1b of **G 2/10**). This is clearly the case, when the embodiment *"imidazolinone resistance"* is removed from the list in paragraph [0016], since it is clear to the skilled person that in this case *"glyphosate and*

glufosinate-resistance" remains. As set out above, all subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 7 of the Main Request fulfils the requirements of Articles 76 (1) and 123(2) EPC.

2.4 Claim 16 of the Main Request fulfils the requirements of Art. 76(1) and Art. 123(2) EPC

2.4.1 The Opponent asserted that the subject-matter of granted claim 15, which corresponds to claim 16 of the Main Request, contravenes the requirements of Articles 76(1) and 123(2) EPC.

The Opponent conceded that the wording of granted claim 15 can be found in the wording of original claims 61 of the parent application, but held that granted claim 15 does not comprise the limited definition of the nucleotide sequence implied by the reference of original claims 61 to original claims 5 and 1.

2.4.2 Claim 61 refers to claim 5 of the parent application, which in turn refers to claim 1. As detailed in sections 2.1.1 to 2.1.6 above, claim 1 together with paragraphs [0050], [0095], [0112], [0124], [0126], [0127], and [0149] of the parent application provide a basis under Art. 76(1) EPC for the subject-matter of claim 1 of the Main Request. Accordingly, the subject-matter of claim 16 of the Main Request finds basis under Art. 76(1) EPC in claims 1 and 61 together with paragraphs [0050], [0095], [0112], [0124], [0126], [0127], and [0149] of the parent application.

As explained in section 2 above, all subject-matter fulfilling the requirements of Art. 76(1) EPC also fulfils the requirements of Art. 123(2) EPC. Accordingly, the subject-matter of claim 16 of the Main Request fulfils the requirements of Articles 76(1) and 123(2) EPC.

2.5 Claims 3-6, 8-15 and 17 of the Main Request fulfil the requirements of Art. 76(1) and Art. 123(2) EPC

2.5.1 The Opponent contended that the subject-matter of granted claims 2-5, 7-14 and 16, which correspond to claims 3-6, 8-15 and 17, respectively, of the Main Request, contravenes the requirements of Art. 76(1) EPC. However, the objections against granted claims 2-5, 7-14 and 16 were only based on the fact that said claims depend on claim 1.

2.5.2 Since the subject-matter of granted claim 1 as well as of claims 1 and 2 of the Main Request complies with the requirements of Art. 76(1) and Art. 123(2) EPC (see section 2.1 and 2.2 above), the objections against dependent claims 3-6, 8-15 and 17 of the Main Request are unfounded.

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3. Entitlement to Priority, Art. 87 EPC

The Opponent held the view that the subject-matter of the granted claims is not entitled to the priority date of April 30, 2004 but only to the international filing date of May 2, 2005 and, therefore regarded **D12** (Bisht *et al.*, 2004) and **D15** (Schleinitz *et al.*, 2004) published after April 30, 2004 but prior to May 2, 2005 as prior art.

The Opponent cites **D12** in an attack on the novelty of the subject-matter of granted claims 1, 11 and 13, which correspond to claims 1/2, 12 and 14 of the Main Request, respectively. The Opponent further cited **D15** in an attack on the inventiveness of the subject-matter of granted claim 4, which corresponds to claim 5 of the Main Request.

Patentee does not regard it as expedient to discuss the validity of the priority claim for each and every claim of the Main Request. The only claims for which it may play a role whether they are entitled to the priority date of April 30, 2004 or not are claims 1, 2, 5, 12 and 14 of the Main Request, which correspond to the claims attacked on the basis of **D12** and **D15** and, thus in the following analysis, it will only be discussed whether the subject-matter of claims 1, 2, 5, 12 and 14 of the Main Request are entitled to the priority date of April 30, 2004.

3.1 Claim 1 of the Main Request is entitled to the priority date of April 30, 2004

The subject-matter of claim 1 of the Main Request has a basis in the priority document US 60/567,052 **D7**.

3.1.1 A basis for the feature *"A transgenic plant cell comprising an isolated polynucleotide that encodes a protein"* can be found at least in paragraph [00123] of the priority document (see page 29 of **D7**).

3.1.2 A basis for the feature *"that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* can be found at least in paragraph [0019] of the priority document (see page 6 of **D7**), especially in the first sentence of paragraph [0019].

3.1.3 A basis for the feature *"wherein said protein is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"* can be found at least in paragraph [00116] of the priority document (see pages 27-28 of **D7**) in combination with paragraphs [0094], [0035], [0036] and [0037] of the priority document (see pages 7-8 of **D7**). The specific numerical value of 70% has a literal basis on page 28, line 2.

3.1.4 A basis for the feature *"wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9"* can be found at least in paragraphs [00100]-[00101] of the priority document (see page 22 of **D7**) in combination with paragraphs [0035] and [0039] of the priority document (see pages 7 and 8 of **D7**). It is explained in paragraph [00100] that

> *"[...] the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene."*

Stringent hybridization conditions are explained in the subsequent paragraph, i.e. in paragraph [00101] on pages 22-23 of **D7**.

One preferred protein sequence of the invention is the protein sequence of SEQ ID NO: 9 (see paragraph [0039]), which is the native amino acid sequence encoded by the *aad-1 (v1)* gene from *Sphingobium herbicidovorans* (see SEQ ID NO: 3 and the explanation in paragraph [0035]).

Taken together, the feature *"wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9"* has a clear basis in the priority document.

3.1.5 Finally, the feature *"wherein said plant expresses said polynucleotide and produces said protein"* has a basis at least in paragraph [00124] of **D7** (see page 29). Said paragraph is part of a section discussing transgenic hosts, especially transgenic plant hosts and transgenic plant cells. The first three sentences of paragraph [00124] read:

> *In preferred embodiments, expression of the gene results, directly or indirectly in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells.*

3.1.6 In conclusion, the subject-matter of claim 1 of the Main Request is fully based on the disclosure of the priority document and entitled to the priority date of April 30, 2004. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 1.

3.2 Claim 2 of the Main Request is entitled to the priority date of April 30, 2004

The subject-matter of claim 2 of the Main Request has a basis in the priority document US 60/567,052 **D7**.

3.2.1 Basis for the features:

- *"A transgenic plant comprising an isolated polynucleotide that encodes a protein"*;
- *"wherein said polynucleotide hybridizes under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO:9"*, and
- *"wherein said plant expresses said polynucleotide and produces said protein"*

has already been provided in section 3.1 above.

3.2.2 A basis for the features *"that enzymatically degrades (i) an R-enantiomer of chiral phenoxy auxin herbicides in addition to achiral phenoxy auxins, (ii) an aryloxyphenoxypropionate herbicide, or (iii) a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide"* can be found at least in paragraphs [0019] and [0051] of **D7** (see pages 6 and 9).

More specifically, paragraph [0019] of **D7** recites that

> *"This invention relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides."*

An almost identical statement can be found in paragraph [0061] of **D7**. Thus, paragraphs [0019] and [0061] provide a basis for the enzyme activities recited as alternatives (ii) and (iii) of claim 2.

Paragraph [0051] of **D7** teaches that

> *"An additional benefit of the AAD-1 gene is that unlike all tfdA homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (e.g., dichlorprop and mecoprop) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid)."*

This paragraph provides a basis for the enzyme activities recited in alternative (i) of claim 2.

3.2.3 In conclusion, the subject-matter of claim 2 of the Main Request is fully based on the disclosure in the priority document and entitled to the priority date of April 30, 2004. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 2.

3.3 Claim 5 of the Main Request is entitled to the priority date of April 30, 2004

3.3.1 Basis for the reference to SEQ ID NO: 9 in claim 5 can be found in paragraph [0040] of **D7** (see page 8). Basis for *"a variant of SEQ ID NO: 9"* can be found in paragraph [00109] of **D7** (see especially page 25, lines 1-3).

In said paragraph it is also indicated that variant proteins have equivalent or similar activity to an exemplified protein (see for example last sentence on page 24 of **D7**). This statement in combination with the information provided in paragraph [0019] provides a basis for the feature *"wherein said variant enzymatically degrades said herbicide"*.

3.3.2 Basis for the feature *"(has) at least one amino acid deletion or conserved substitution"* can be found in paragraph [00109] of the priority document. Conservative amino acid substitutions are explained in detail in paragraph [00118] and Table 5 of the priority document.

3.3.3 Basis for the feature *"(has) at least 95% sequence identity with SEQ ID NO: 9"* can be found in paragraph [00116] of the priority document. The value of 95% has an explicit basis in the penultimate line of page 27 and in the third line of page 28. As already mentioned above, a basis for the reference to SEQ ID NO: 9 can be found in paragraph [0040] of the priority document (see page 8 of **D7**).

3.3.4 In conclusion, the subject-matter of claim 5 of the Main Request is fully based on the disclosure in the priority document and is entitled to the priority date of April 30, 2004. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 5.

3.4 Claim 12 of the Main Request is entitled to the priority date of April 30, 2004

There is ample support for the subject-matter of claim 10 (i.e. the herbicide is a phenoxy auxin, preferably a 2,4-D herbicide) in the priority document, e.g. in paragraph [0015], first sentence; and in paragraphs [0017], [0051], [0064], and [0074] of **D7**.

Accordingly, the subject-matter of claim 12 of the Main Request is fully based on the disclosure in the priority document and is entitled to the priority date of April 30, 2004. Hence **D12** and **D15** do not constitute prior art for the subject-matter of claim 12.

3.5 Claim 14 of the Main Request is entitled to the priority date of April 30, 2004

It is disclosed in paragraph [0019] that aryloxyalkanoate dioxygenase enzymes of the invention are *"capable of degrading phenoxy auxin and aryloxyphenoxypropionate (AOPP) herbicides"*. It is further disclosed in paragraph [0015] of the priority document that the *"subject invention provides novel plants that are not only resistant to 2,4-D, but also to AOPP herbicides"*. The abbreviation 2,4-D is explained in paragraph [0006] and refers to 2,4-dichlorophenoxyacetic acid. It is explained in paragraph [0014] that haloxyfop, quizalofop and diclofop are AOPP herbicides. It is further indicated in paragraph [0051] that MCPA, dichlorprop, and mecoprop are phenoxy auxin herbicides (see page 9, lines 5-8 of **D7**). As mentioned in paragraph [00243], quizalofop, cyhalofop and haloxyfop are AOPP herbicides (see paragraph [00243], especially

page 64, lines 6-8 of **D7**). Finally, paragraph [00313] of **D7** contains the information that fluazifop, haloxyfop, quizalofop, and fenoxaprop are AOPP herbicides.

Accordingly, the herbicides listed have a basis in the priority document and are entitled to the priority date of April 30, 2004. Therefore, **D12** and **D15** do not constitute prior art for these herbicides.

3.6 Summarizing, at least the subject-matter of granted claims 1, 2, 5, 12 and 14 of the Main Request is entitled to the priority date of April 30, 2004. Accordingly, **D12** and **D15** do not form prior art for the subject-matter of these claims.

4. **Novelty; Articles 54 and 100(a) EPC**

The Opponent raised novelty objections against the subject-matter of granted claims 1, 11 and 13, which correspond to claims 1/2, 12 and 14 of the Main Request, respectively. The subject-matter of these claims was said to be anticipated by each one of **D8**, **D9**, **D10**, **D11**, and **D12**, respectively.

These five novelty attacks are based on an identical line of argumentation, since all of **D8** to **D12** refer to transgenic dicot plants and plant cells comprising the *tfdA* gene from *Ralstonia eutropha* (previous name: *Alcaligenes eutrophus*; later renamed as: *Wautersia eutropha*; current name: *Cupriavidus necator*). The Opponent has submitted results of experiments as **D1** which allegedly demonstrate that the *tfdA* gene hybridizes under "stringent conditions" with the polynucleotide recited in claim 1. The Opponent further pointed out that the TfdA protein has 2,4-D degrading activity, which means that the TfdA protein degrades a phenoxy auxin herbicide as required in one alternative by granted claim 1.

In the enclosed Main Request claim 1 was split into two independent claims. In amended claim 1, the encoded protein is further defined in structural terms. In amended claim 2, the encoded protein is further defined in functional terms.

4.1 The subject-matter of amended claim 1 is novel over the prior art

4.1.1 Without acquiescing to Opponent's assertion that the results of the experiments submitted as **D1** actually show that polynucleotides encoding TfdA hybridizes **under stringent conditions with the full complement of a nucleic acid molecule that encodes SEQ ID NO: 9** Patentee has decided to address this objection by amendments. Accordingly, amended claim 1 further characterizes the protein encoded by the isolated polynucleotide present within the transgenic plant cell in that it is

"at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5"

4.1.2 The Opponent has carried out a sequence alignment between the protein encoded by the *tfd*A gene of *Ralstonia eutropha* and the *aad-1* gene of the Opposed Patent (see **D4**). According to this sequence alignment the protein encoded by the *tfd*A gene of *Ralstonia eutropha* exhibits about 32% sequence identity to the protein encoded by the *aad-1* gene of the Opposed Patent. The polynucleotide sequence according to SEQ ID NO: 3 encodes the wild type AAD-1 protein according to SEQ ID NO: 9. The polynucleotide sequence according to SEQ ID NO: 4 encodes the AAD-1 protein according to SEQ ID NO: 10, which only differs from wild type AAD-1 in an Arg to Cys mutation at position 212 and a His to Ala mutation at position 2. The polynucleotide sequence according to SEQ ID NO: 5 encodes the AAD-1 protein according to SEQ ID NO: 11, which only differs from wild type AAD-1 protein by an additional Ala that is inserted between the N-terminal Met and the His at position 2. In its alignment Opponent appears to have used the wild type AAD-1 amino acid sequence according to SEQ ID NO: 9 (it is noted that position 212 of the query sequence is Arg and not Cys and that position 15 is Ile which is only the case in the amino acid sequence according to SEQ ID NO: 9). Accordingly, the alignment with either the protein according to SEQ ID NO: 10 or 11 will lead to an even lower level of sequence identity than 32%.

4.1.3 Claim 1 of the Main Request requires that the transgenic plant cell comprises a polynucleotide encoding a protein that is at least 70% identical with a protein encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, i.e. the proteins with the amino acid sequences according to SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11, respectively. As explained above, the protein encoded by the *tfdA* gene from *Ralstonia eutropha* exhibits 32% or less sequence identity to the amino acid sequences of SEQ ID NO: 9, 10 or 11. Therefore, the transgenic plant cells described in **D8**, **D9**, **D10**, **D11**, and **D12** do not anticipate the subject-matter of amended claim 1 and hence the subject-matter of claim 1 of the Main Request is novel over the prior art.

For the sake of completeness, it is noted again that **D12** does not even constitute prior art for the subject-matter of amended claim 1 of the Main Request (see section 3.1 above).

4.2 The subject-matter of amended claim 2 is novel over the prior art

4.2.1 Amended claim 2 characterizes the protein encoded by the isolated polynucleotide present within the transgenic plant in that it

enzymatically degrades

(i) an R-enantiomer of chiral phenoxy auxin herbicide in addition to achiral phenoxy auxins,

(ii) an aryloxyphenoxypropionate herbicide, or