Supplemental Data

Supplemental Figure 1: Gene structures of MAM genes identified in diploid Brassica species. (Supports Figure 2a). A-, B-, and C- genomes correspond to *B. rapa*, *B. nigra*, and *B. oleracea*. Sizes (in bp) and organization of exons (dark boxes) and introns (lines) of each *MAM* gene are drawn to scale.



Supplemental Figure 2: Expression and purification of recombinant MAMS from *A. thaliana* and *B. juncea.* (Supports Figures 4 and 5). AtMAM1 and the four BjMAM proteins were expressed and purified from *E. coli*, as described in the Methods. SDS-PAGE showing Ni²⁺-affinity purified AtMAM1, BjMAM1-A, BjMAM2-A, BjMAM1-B, and BjMAM2-B. Equal amounts of protein (2 μ g) were loaded, separated, and stained using Coomassie Blue. Molecular weight marker (M) is in the first lane.



Supplemental Figure 3: Analysis of 2-malate derivatives by LC-MS/MS. (Supports Figures 2c-d and 5). Assays were performed as described in the Methods. A sample (1 μ L) from each assay was analyzed by LC-MS/MS to quantify resulting products. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies) using a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 μ m, Agilent). Detection in MRM mode with an API3200 triple quadrupole mass spectrometer (Applied Biosystems). Abbreviations: 2-MTEM, 2-(2'-methylthio)ethylmalate; 2-MTPM, 2-(2'-methylthio)propylmalate; 2-MTBM, 2-(2'-methylthio)butylmalate.



Time, min

Supplemental Figure 4: Expression of *B. juncea MAM* genes in 5-day old T2 seedlings of selected BjMAM1-A(as) and BjMAM2-A(as) lines. (Supports Figure 2d). Transcript accumulation was measured for *BjMAM1-A*, *BjMAM1-B*, *BjMAM2-A* and *BjMAM2-B* genes. The qRT-PCR reactions were performed and the data represent mean of two independent experiments, with similar trends. The heat map was constructed based on the expression value normalized against the *B. juncea actin* (*BjACTIN*) gene expression level, and expression in wild-type (WT) was set at 1. For the qRT-PCR analysis, the forward (F) and reverse (R) primers were as follows: BjMAM1-A-F 5'-ATCCCTACCACCGGTTCGAA-3'; BjMAM1-A-R 5'-GAGCTTGTTCGGAATGTACTCC-3'; BjMAM2-A-F 5'-TCATGTTGCTCCCCAGTGTCT-3'; BjMAM2-A-R 5'-CGGAGTTTGCGAGCTG TCTA-3'; BjMAM1-B-F 5'-GCAAACACTCCTGGCATTGAC-3'; BjMAM1-B-R 5'-TGAATGCCGCT CTCATGAACA-3'; BjMAM2-B-F 5'-AGGGGGAGACTAGTGCGAGTGG-3'; BjMAM2-B-R 5'-AGA CGACGCCGGAAAACCG-3'; BjActin-F 5'-CTTCTTACCGAGGCTCCTCT-3'; BjActin-R 5'-AAGG ATCTTCATGAGGTAATCAGT-3'.



Supplemental Figure 5: BjMAM1-A domain comparison and ligand binding. (Supports Figure 4). (a) Comparison of the three-dimensional structures of BjMAM1-A and IPMS from *Mycobacterium tuberculosis*. The structures of the BjMAM1-A (α -helices, rose and β -strands, blue) and *M. tuberculosis* IPMS (PDB: 1SR9; α -helices, gold and β -strands, green) monomers are superimposed. Conserved features, including the N-terminal α/β -barrel domain and the α -helical region that extends to form part of the CoA binding site are indicated. Regions that differ, including the extended N-terminal and C-terminal regulatory domain of IPMS, are indicated with italic text. (b) Electron density for 4MTOB (left) and CoA (right) in the BjMAM1-A active site is shown as a 2Fo-Fc omit map (1.5 σ). (c) Schematic of interactions in the 4MTOB binding site. For panels c and e, interactions are shown as dotted lines with distances indicated in Å. (d) Active site entrance and CoA binding site. A protein surface is shown with portions corresponding to each chain of the dimer shown in gray (upper) and white (lower), respectively. CoA is shown as a stick model. Note the pantothenate arm extending into the structure toward the buried catalytic site. (e) Schematic of interactions in the CoA binding site.



Supplemental Table 1: Summary of MAM genes from *A. thaliana* **and the cultivated** *Brassica*** species.** (Supports Figure 2a-b). Gene IDs were retrieved from the BRAD database (Brassicadb.org/brad). Gene, CDS, exon, and intron sizes are in number of base pairs (bp); protein size is number of amino acids.

Gene AtMAM1	Gene 3597	CDS 1521	Protein 506	Exons (size of each) 10(444, 260, 214, 81, 93, 87, 101, 34, 75, 132)	Introns (size of each) 9 (677, 501, 130, 90, 86, 204, 92, 102, 194)	BRAD ID At5g023010
B. rapa (A	genome)				
BrMAM1	4240	1518	505	10 (432, 260, 214, 81, 93, 87, 101, 34, 75, 141)	9 (1349, 537, 99, 166, 83, 94, 121, 117, 156)	Bra029355
BrMAM2	2785	1515	504	10 (444, 260, 214, 81, 93, 87, 101, 34, 75, 126)	9 (205, 224, 93, 174, 100, 81, 91, 134, 168)	Bra013007
B. nigra (B	genome	e)		-)		
BnMAM1	3191	1518	504	10 (432, 260, 214, 81, 93, 87, 101, 34, 75, 141)	9 (506, 425, 87, 83, 96, 100, 102, 123, 151)	BniB003309
BnMAM2	3004	1518	504	10 (447, 260, 214, 81, 93, 87, 101, 34, 75, 123)	9 (1 ⁸ 2, 282, 90, 174, 96, 92, 96, 335, 139)	BniB000352
B. oleracea	a (C aen	ome))		
BoMAM1	4222	1518	505	10 (432, 260, 214, 81, 93, 87, 101, 34, 75, 141)	9 (1345, 468, 109, 187, 83, 94, 118, 125, 175)	Bol020647
BoMAM2	2795	1527	508	10 (444, 260, 214, 81, 93, 87, 101, 34, 78, 135)	9 (215, 214, 95, 168, 105, 90, 83, 159, 139)	Bol017070
B. juncea (AB geno	ome)				
BjMAM1- A	4260	1518	505	10 (432, 260, 214, 81, 93, 87, 101, 34, 75, 141)	9 (1349, 536, 99, 165, 83, 94, 121, 117, 178)	BjuA033169
BjMAM1- B	3266	1518	505	10 (432, 260, 214, 81, 93, 87, 101, 34, 75, 141)	9 (496, 423, 88, 187, 83, 96, 100, 123, 152)	BjuB017494
BjMAM2- A	2778	1515	504	10 (444, 260, 214, 81, 93, 87, 101, 34, 75, 126)	9 (205, 229, 93, 175, 87, 81, 91, 134, 168)	BjuB020681
BjMAM2- B	3000	1512	503	10 (447, 260, 214, 81, 93, 87, 101, 34, 75, 120)	9 (182, 282, 90, 174, 96, 93, 96, 336, 139)	BjuB037742

Supplemental Table 2: Details of the analysis of malate derivatives by LC-MS/MS using an Agilent HPLC 1200/API-3200 instrument in negative ionisation mode. (Supports Figure 2c-d). Abbreviations are: Q1, selected *m/z* of the first quadrupole; Q3, selected *m/z* of the third quadrupole; RT, retention time; DP, declustering potential (V); and CE, collision energy (V).

Compound	Q1	Q3	RT (min)	DP	CE
2-isopropyl-malate	175	115	2.68	-30	-20
2-(2-methylthioethyl)-malate	207	147	2.67	-30	-20
2-(3-methylthiopropyl)-malate	221	161	2.91	-30	-20
2-(4-methylthiobutyl)-malate	235	175	3.18	-30	-20
2-hexyl-malate	217	157	3.76	-30	-20
2-heptyl-malate	231	171	4.05	-30	-20
2-(6-methylthiohexyl)-malate	263	203	n.d.	-30	-20
2-(7-methylthioheptyl)-malate	277	217	n.d.	-30	-20
2-decyl-malate	273	213	n.d.	-30	-20

Supplemental Table 3: Average glucosinolate content and profile in T2 seeds of various lines harboring *MAM* constructs in the *A. thaliana MAM1* knockout line (*TU1*) background. (Supports Figure 2c). The glucosinolate (GSL) profiles of the transgenic lines were estimated using HPLC, as described in the Methods. Values (μ mol g⁻¹ dry weight) are shown as mean ± SE of three independent transformants. GSL are as follows: C3-GSL are 3MSOP, 3MTP, 3OHP, 3BzOP; C4-GSL are 4MSOB, 4MTB, 4OHB, 4BZOB; C5-GSL are 5MSOP, 5MTP; long-chain GSL are 6MSOH, 7MSOH, 8MSOO, 7MTH, 8MTO; and indole GSL are I3M and 4MOI3M.

Line	C3-GSL	C4-GSL	C5-GSL	Long-chain-GSL	Indole-GSL	Total GSL
Col-0 (WT)	3.7 ± 0.1	45.9 ± 5.2	0.6 ± 0.2	18.8 ± 0.3	3.2 ± 1.1	72.3 ± 3.9
TU1 mutant	35.9 ± 0.9	0.9 ± 0.2	0.0 ± 0.0	21.5 ±1.9	2.0 ± 0.2	60.5 ± 2.4
vector (TU1)	32.0 ± 0.9	0.7 ± 0.1	0.0 ± 0.0	20.7 ± 1.3	3.3 ± 0.6	56.9 ± 2.6
AtMAM1 (TU1)	23.2 ± 4.2	39.2 ± 3.8	0.2 ± 0.1	17.7 ± 0.7	3.1 ± 0.3	83.5 ± 3.9
BjMAM1-A (TU1)	15.5 ± 2.6	42.1 ± 5.8	0.6 ± 0.1	19.7 ± 2.3	2.0 ± 0.2	80.0 ± 10.5
BjMAM2-A (TU1)	67.5 ± 8.5	3.0 ± 1.2	0.0 ± 0.0	20.0 ± 2.3	4.0 ± 1.5	94.6 ± 8.1

Supplemental Table 4: Average glucosinolate content and profile in seeds of wild-type *B. juncea* (cv. Varuna) and BjMAM antisense transgenic *B. juncea* lines. (Supports Figure 2d). *B. juncea* was transformed with either construct C9 [ptNative:BjMAM1-A(as)] or C10 [ptNative:BjMAM2-A(as)]. Values (μ mol g⁻¹ dry weight) of T2 seeds obtained from at least five independent T1 progreny are shown as mean ± SE. The percentages of C3-, C4- and C5-glucosinolates (GSL) of total aliphatic GSL are summarized. GSL are as follows: C3-GSL: sinigrin, 3MSOP; C4-GSL: 3-butenyl, 4MSOB; C5-GSL: 4-pentenyl.

Line	C3-GSL	C4-GSL	C5-GSL	Total GSL	C3/Total (%)	C4/Total (%)	C5/Total (%)
WT	14.9 ± 0.9	65.7 ± 4.4	7.5 ± 0.3	88.1 ± 5.5	16.9 ± 1.1	74.6 ± 5.0	8.5 ±0.3
ptNative:	BjMAM1-A(a	is)					
1-Aas-1	25.7 ± 1.3	70.5 ± 1.4	7.8 ± 0.6	104 ± 2	24.7 ± 1.2	67.8 ±1.3	7.5 ± 0.5
1-Aas-2	33.4 ± 2.4	41.1 ± 4.6	9.6 ± 0.2	84.1 ± 3.0	39.7 ± 2.9	48.9 ±5.5	11.4 ± 0.2
1-Aas-3	34.6 ± 3.2	62.4 ± 2.9	6.8 ± 0.9	104 ± 2	33.3 ± 3.1	60.1 ± 2.8	6.6 ± 0.8
1-Aas-5	25.1 ± 2.3	74.9 ± 2.7	8.0 ± 0.5	86.4 ± 21.9	23.2 ± 2.1	69.3 ± 2.5	7.5 ± 0.4
1-Aas-6	23.0 ± 1.0	65.3 ± 1.3	7.0 ± 0.6	95.3 ± 2.2	24.1 ± 1.0	68.5 ±1.3	7.4 ± 0.6
1-Aas-7	21.0 ± 2.6	69.9 ± 2.9	6.9 ± 0.3	97.8 ± 5.1	21.5 ± 2.7	71.5 ± 2.9	7.0 ± 0.3
1-Aas-10	40.4 ± 4.0	65.0 ± 1.5	5.5 ± 0.6	111 ± 5	36.4 ± 3.6	58.6 ± 1.4	5.0 ± 0.5
1-Aas-12	18.8 ± 1.1	63.2 ± 1.4	7.4 ± 0.2	89.5 ± 1.2	21.1 ± 1.2	70.6 ± 1.5	8.3 ± 0.2
1-Aas-16	29.5 ± 0.7	60.1 ± 1.4	6.7 ± 0.2	96.3 ± 1.8	30.6 ± 0.8	62.4 ± 1.4	7.0 ± 0.2
1-Aas-17	25.6 ± 1.5	53.4 ± 4.5	7.1 ± 0.4	86.1 ± 2.8	29.7 ± 1.8	62.1 ± 5.1	8.2 ± 0.5
1-Aas-18	25.6 ± 2.0	62.0 ± 1.4	6.5 ± 0.1	94.1 ± 2.1	27.2 ± 2.2	65.9 ± 1.5	6.9 ± 0.1
1-Aas-19	37.3 ± 1.8	25.6 ± 1.5	9.0 ± 0.2	71.9 ± 2.9	51.9 ± 2.2	35.5 ± 1.8	12.6 ± 0.2
1-Aas-20	29.9 ± 3.2	55.9 ± 5.3	6.7 ± 0.4	92.6 ± 3.8	32.4 ± 3.2	60.4 ± 5.1	7.2 ± 0.4
1-Aas-21	18.4 ± 0.9	66.3 ± 0.8	6.8 ± 0.2	91.5 ± 1.3	20.1 ± 0.9	72.5 ± 0.8	7.4 ± 0.2
1-Aas-22	25.9 ± 3.1	62.6 ± 2.6	5.6 ± 0.2	94.2 ± 1.1	27.6 ± 2.9	66.4 ± 2.4	6.0 ± 0.2
1-Aas-23	27.0 ± 2.0	55.0 ± 3.1	7.1 ± 0.4	90.1 ± 2.0	30.0 ± 2.0	62.1 ± 3.0	7.9 ± 0.4
1-Aas-25	24.4 ± 1.5	74.3 ± 2.5	6.5 ± 0.3	105 ± 3	23.2 ± 1.3	70.6 ± 2.1	6.2 ± 0.3
1-Aas-26	24.5 ± 4.7	66.4 ± 4.0	6.8 ± 0.4	97.6 ± 4.8	25.1 ± 4.9	68.0 ± 4.0	6.9 ± 0.4
1-Aas-27	26.2 ± 5.3	63.0 ± 7.2	3.9 ± 0.7	93.1 ± 9.8	28.2 ± 5.6	67.6 ± 7.7	4.2 ± 0.8
1-Aas-28	16.2 ± 0.8	72.6 ± 1.9	6.6 ± 0.2	95.3 ± 2.4	17.0 ± 0.8	76.1 ± 2.0	6.9 ± 0.2
ptNative:	BjMAM2-A(a	is)					
2-Aas-1	19.7 ± 0.5	72.9 ± 0.7	7.0 ± 0.3	99.6 ± 1.0	19.8 ± 0.5	73.2 ± 0.7	7.0 ± 0.8
2-Aas-3	16.9 ± 0.6	68.3 ± 1.9	7.3 ± 0.3	92.4 ± 2.5	18.3 ± 0.7	73.8 ± 2.0	7.9 ± 0.3
2-Aas-4	18.7 ± 2.2	71.7 ± 2.1	8.2 ± 0.4	98.7 ± 3.9	19.0 ± 2.3	72.7 ± 2.1	8.3 ± 0.4
2-Aas-5	16.6 ± 2.1	60.3 ± 2.1	7.4 ± 0.3	84.3 ± 2.2	19.7 ± 2.7	71.5 ± 2.5	8.8 ± 0.3
2-Aas-6	12.9 ± 0.6	58.5 ± 1.9	7.1 ± 0.2	78.4 ± 2.6	16.4 ± 0.8	74.6 ± 2.4	9.0 ± 0.3
2-Aas-7	13.7 ± 0.5	55.0 ± 0.6	6.7 ± 0.2	78.3 ± 0.7	17.4 ± 0.6	74.0 ± 0.8	8.6 ± 0.3
2-Aas-8	18.4 ± 0.8	57.0 ± 4.1	7.4 ± 0.1	82.8 ± 4.0	22.2 ± 1.0	68.8 ± 5.0	9.0 ± 0.1
2-Aas-9	18.9 ± 0.7	53.8 ± 1.7	7.5 ± 0.3	80.2 ± 2.0	23.6 ± 0.8	67.1 ± 2.0	9.3 ± 0.3
2-Aas-10	15.6 ± 0.4	62.7 ± 1.2	7.0 ± 0.2	85.3 ± 1.4	18.3 ± 0.5	73.5 ± 1.5	8.2 ± 0.2
2-Aas-11	20.4 ± 3.1	63.3 ± 4.4	7.3 ± 0.1	90.9 ± 7.4	22.5 ± 3.4	69.5 ± 4.8	8.0 ± 0.1
2-Aas-12	17.4 ± 0.6	58.8 ± 1.9	6.7 ± 0.1	82.9 ± 1.6	20.9 ± 0.7	71.0 ± 2.3	8.1 ± 0.2
2-Aas-13	15.8 ± 0.4	58.8 ± 0.9	7.2 ± 0.2	81.8 ± 1.0	19.3 ± 0.5	71.9 ± 1.0	8.9 ± 0.3
2-Aas-14	15.7 ± 0.7	64.0 ± 1.5	7.6 ± 0.2	87.2 ± 1.9	18.0 ± 0.8	73.3 ± 1.7	8.7 ± 0.2
2-Aas-15	14.8 ± 0.8	61.6 ± 2.3	6.1 ± 0.2	82.5 ± 3.0	17.9 ± 1.0	74.8 ± 2.7	7.3 ± 0.3

Supplemental Table 5: ANOVA Statistical Analysis

Figure 2C: ANOVA table for %C3/C3+C4 and %C4/C3+C4 glucosinolate (GSL) fractions

	Sum of Squares (sq)	Degrees of freedom (df)	Mean Square	F-value	<i>P</i> -value			
Between Groups	25359.125	5	5071.825	664.433	0.000			
Within Groups	91.600	12	7.633					
Total	25450.724	17						

Multiple comparisons (LSD) for %C3/C3+C4 GSL fraction

	Complement ed Lines (CL)	Mean difference (TU1-CL)	Std. Error	<i>P</i> -value	Lower Bound	Upper bound
TU1	Col	89.91055	2.25585	0.0000	84.9955	94.8256
	VC	-0.2057	2.25585	0.9290	-5.1208	4.7094
	AtMAM1	60.23833	2.25585	0.0000	55.3232	65.1534
	BjMAM1-A	70.54781	2.25585	0.0000	65.6327	75.4629
	BjMAM2-A	1.86033	2.25585	0.4260	-3.0548	6.7754

Multiple comparisons (LSD) for %C4/C3+C4 GSL fraction

	Complement ed Lines (CL)	Mean difference (TU1-CL)	Std. Error	<i>P</i> -value	Lower Bound	Upper bound
TU1	Col	-89.91055	2.25585	0	-94.8256	-84.9955
	VC	0.2057	2.25585	0.929	-4.7094	5.1208
	AtMAM1	-60.23833	2.25585	0	-65.1534	-55.3232
	BjMAM1-A	-70.54781	2.25585	0	-75.4629	-65.6327
	BjMAM2-A	-1.86033	2.25585	0.4260	-6.7754	3.0548

	Sum of Squares (sq)	Degrees of freedom (df)	Mean Square	F-value	<i>P</i> -value			
Between Groups	8153.815	35	232.966	10.624	0.000			
Within Groups	2916.535	133	21.929					
Total	11070.349	168						

Figure 2D: ANOVA table for total C3-glucosinolate (GSL) in BjMAM1-A(as) and BjMAM2-A(as) lines

Multiple comparisons (LSD) for BjMAM1-A(as) lines

		Mean				
	BjMAM1-A	difference	Std.		Lower	
	(as) lines	(WT-1Aas)	Error	P-value	Bound	Upper bound
WT	1-Aas-1	-10.82775	3.14133	0.0010	-17.0412	-4.6143
	1-Aas-2	-18.51006	3.14133	0.0000	-24.7235	-12.2966
	1-Aas-3	-19.68124	3.14133	0.0000	-25.8947	-13.4678
	1-Aas-5	-10.22496	3.31126	0.0020	-16.7745	-3.6754
	1-Aas-6	-8.08641	3.14133	0.0110	-14.2998	-1.873
	1-Aas-7	-6.14216	3.31126	0.0660	-12.6917	0.4074
	1-Aas-10	-25.56736	3.14133	0.0000	-31.7808	-19.3539
	1-Aas-12	-3.9509	3.14133	0.2110	-10.1643	2.2625
	1-Aas-16	-14.61493	3.14133	0.0000	-20.8284	-8.4015
	1-Aas-17	-10.71953	3.14133	0.0010	-16.933	-4.5061
	1-Aas-18	-10.70116	3.31126	0.0020	-17.2507	-4.1516
	1-Aas-19	-22.38336	3.14133	0.0000	-28.5968	-16.1699
	1-Aas-20	-15.06208	3.14133	0.0000	-21.2755	-8.8486
	1-Aas-21	-3.52115	3.14133	0.2640	-9.7346	2.6923
	1-Aas-22	-11.06244	3.14133	0.0010	-17.2759	-4.849
	1-Aas-23	-11.82146	3.14133	0.0000	-18.0349	-5.608
	1-Aas-25	-9.52558	3.14133	0.0030	-15.739	-3.3121
	1-Aas-26	-9.62793	3.31126	0.0040	-16.1775	-3.0784
	1-Aas-27	-11.34448	3.31126	0.0010	-17.894	-4.7949
	1-Aas-28	-1.27287	3.14133	0.6860	-7.4863	4.9406

Multiple comparisons (LSD) for BjMAM2-A(as) lines

		Mean				
	BjMAM2-A	difference	Std.		Lower	
	(as) lines	(WT-2Aas)	Error	P-value	Bound	Upper bound
WT	2-Aas-1	-4.95752	3.31126	0.1370	-11.5071	1.592
	2-Aas-3	-2.00622	3.14133	0.5240	-8.2197	4.2072
	2-Aas-4	-3.86823	3.14133	0.2200	-10.0817	2.3452
	2-Aas-5	-1.59729	3.14133	0.6120	-7.8107	4.6161
	2-Aas-6	2.02926	3.14133	0.5190	-4.1842	8.2427
	2-Aas-7	1.27216	3.14133	0.6860	-4.9413	7.4856
	2-Aas-8	-3.54217	3.14133	0.2620	-9.7556	2.6713
	2-Aas-9	-3.9569	3.14133	0.2100	-10.1703	2.2565
	2-Aas-10	-0.76693	3.14133	0.8070	-6.9804	5.4465
	2-Aas-11	-5.54632	3.31126	0.0960	-12.0959	1.0032
	2-Aas-12	-2.46927	3.31126	0.4570	-9.0188	4.0803
	2-Aas-13	-0.89312	3.14133	0.7770	-7.1066	5.3203
	2-Aas-14	-0.80841	3.57657	0.8220	-7.8827	6.2659
	2-Aas-15	0.09268	3.14133	0.9770	-6.1208	6.3061