

Supplementary Information

An histidine covalent receptor/butenolide complex mediates strigolactone perception

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Supplementary Results

Supplementary Table 1. List of protein sequences used in the phylogenetic analysis (see **Supplementary Figure 2**) and in protein alignment (see **Supplementary Figure 4**).

Sequence ID	NCBI Ref number	Genebank GI number
PsRMS3	KT321518	GI:1839264
MtD14	XP_003589086	GI:357437621
PhDAD2	J9U5U9	GI:476007203
AtD14	NP_566220	GI:18396732
OsD14	NP_001049306	GI:115451411
SbD14	XP_002468316	GI:242041843
PtD14a	XP_002302409	GI:224067208
MtHTLa	XP_003597120	GI:357453679
MtHTLb	AFK41000	GI:388505868
AtHTL	NP_195463	GI:15235567
PtHTL	XP_006380654	GI:566180525
OsHTL	NP_001050445	GI:115453689
SbHTL	XP_002465088	GI:242035387
BsRsbQ	WP_042975329	GI:757754288

(At) *Arabidopsis thaliana* ; (Bs) *Bacillus subtilis* ; (Ph) *Petunia hybrida* ;
(Pt) *Populus trichocarpa* ; (Ps) *Pisum sativum* ; (Mt) *Medicago truncatula* ;
(Os) *Oryza sativa* Japonica Group ; (Sb) *Sorghum bicolor*.

Supplementary Table 2. Sequence analysis of *PsD14* in the *rms3* mutants.

Mutant line	Background	Nucleotide substitution with position	Amino acid substitution with position	Major effect
<i>rms3-1</i>	Torsdag	TGG → TGA (+723)	Trp →STOP (241)	Truncated protein (241 amino acid) Intron is not spliced.
<i>rms3-2</i>	Torsdag	AGG → AGA (+372)	-	Truncated protein (124 amino acid) (see Supplementary Figure 3a)
<i>rms3-3</i>	Raman	GGC → GAC (+44)	Gly →Asp (15)	Protein destabilization (see Supplementary Figure 3a)
<i>rms3-4</i>	T��r��se	GGC → GAC (+83)	Gly →Asp (28)	Protein destabilization (see Supplementary Figure 3b)
<i>rms3-5</i>	T��r��se	TCC → TTC (+287)	Ser →Phe (96)	Abolish catalytic triad

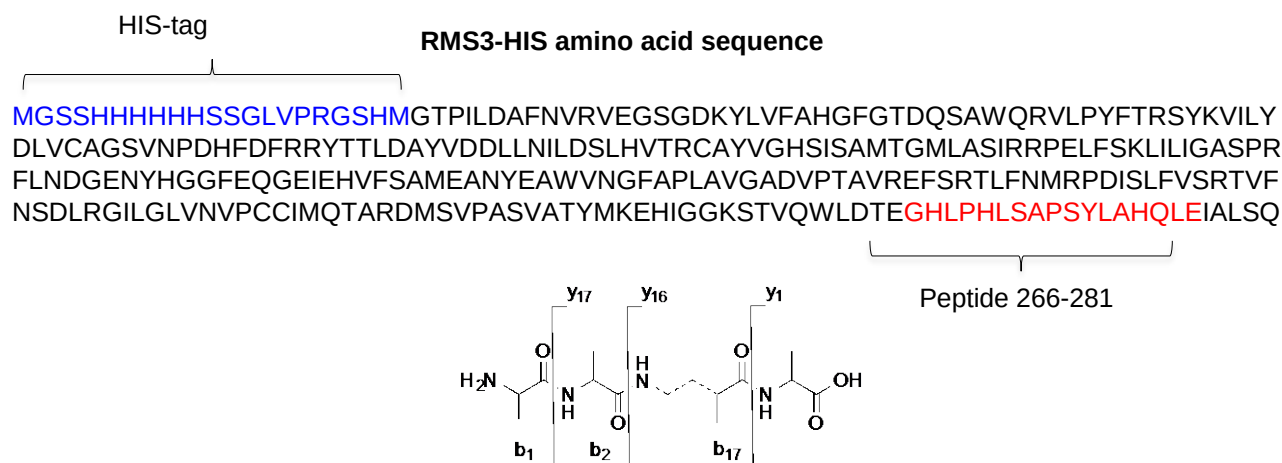
The positions of nucleotide substitutions are labeled from the start of translation (1). Amino acid are numbered from the first Methionine encoded by start codon.

Supplementary Table 3. Steady state kinetic constants for RMS3, RMS3^{S96A}, RMS3^{S96C}, AtD14, AtHTL and AtMES9 with *para*-nitrophenyl acetate (*p*-NPA).

	RMS3	RMS3 ^{S96A}	AtHTL	AtMES9	RMS3 ^{S96C}		AtD14
$V_{\max}$ ($\mu\text{M} \cdot \text{min}^{-1}$)	6.33 ± 0.6	n.d.	57.59 ± 0.20	161.00 ± 2.30			
K_M (μM)	2,412 ± 390	n.d.	2,491 ± 163	1,918 ± 311			
k_{cat} (min^{-1})	3.97 ± 0.32	n.d.	36.00 ± 1.22	8,050 ± 622	V/K (min^{-1})	0.001020 ± 0.000020	0.000850 ± 0.000015
$\text{Eff} = k_{\text{cat}}/K_M$ ($\mu\text{M}^{-1} \cdot \text{min}^{-1}$)	0.0016	n.d.	0.014	4.19			

n.d. : not determined. For RMS3^{S96C} and AtD14 only V/K was determined because they showed linear kinetics (see Supplementary Figure 10). Values represent the mean (±SE) of three replicates and the experiments were repeated at least two times.

Supplementary Table 4. MS/MS data for localization of the amino acid tagged with the D ring on RMS3-HIS. Theoretical fragments detected after MALDI-TOF/TOF-CID-MS/MS for the native (266-281) and modified peptides on histidine 2, produced by endoprotease GluC cleavage. In red: y fragments found in the spectrum of the unmodified peptide (MW 1,870 Da) and common in the two complexes (1,966 and 1,980 Da). In green and blue: b series fragments detected specific of each complexes. In brown: y fragments detected specific of GR24 and GC242 complexes. The shifts of 96 Da and 110 Da observed on the two b series of the peptides modified respectively with GR24 or GC242, demonstrate the fixation of the ligand in the two first amino-acid of the N terminus part of the peptide. The presence of the two peaks at 1,908.9 Da and 1,922.9 Da attests the presence of the ligand on the histidine 2.



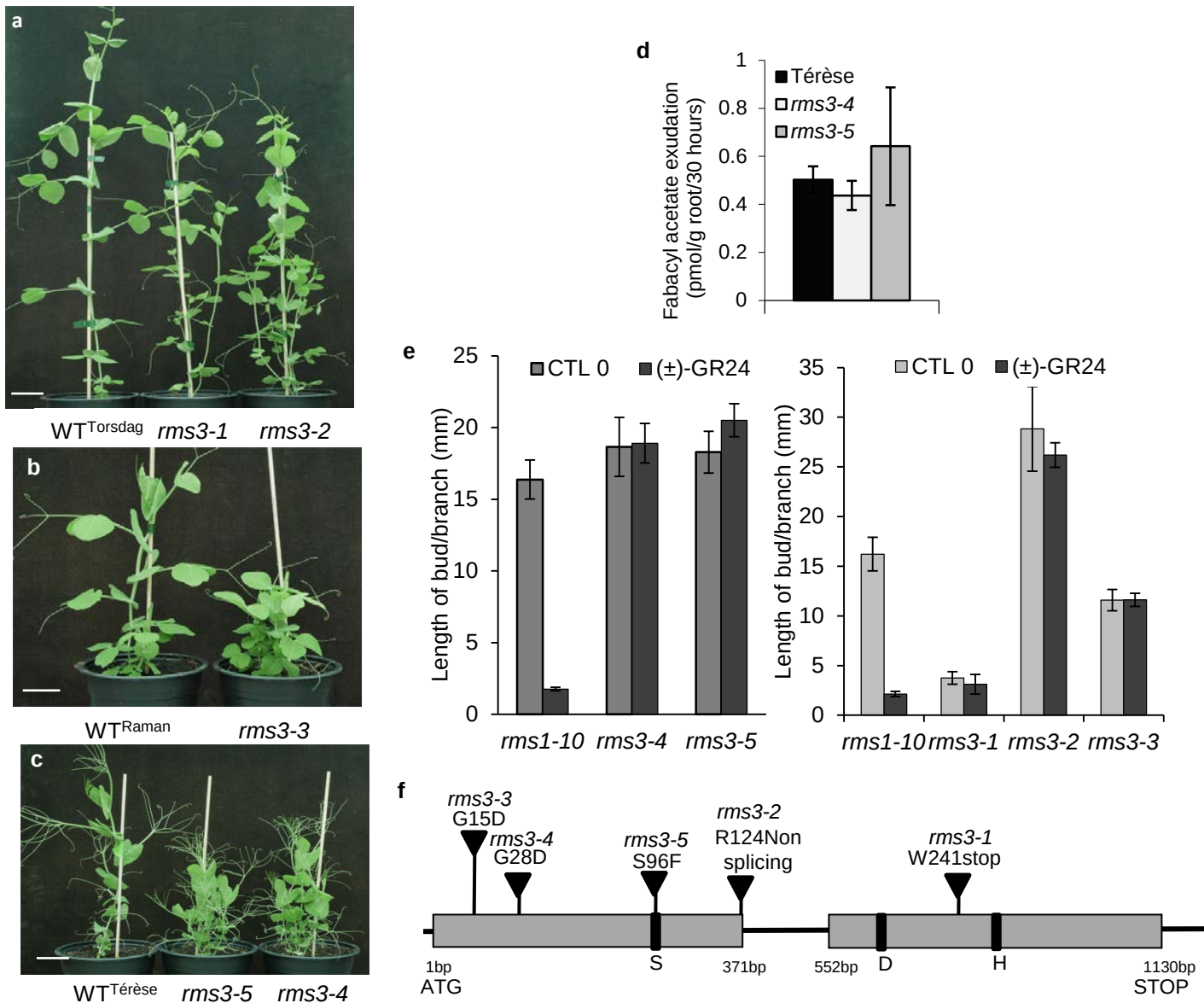
Position	Amino acid	PEPTIDE 266-281 MW=1,870 Da		Complex with (±)-GR24 MW=1,966 Da		Complex with (±)-GC242 MW=1,980 Da	
		b	y	b	y	b	y
1	G	58.03		58.03	-	58.03	
2	H*	195.09	1,812.94	291.09	1,908.94	305.09	1,922.94
3	L	308.17	1,675.88	404.17	1,675.88	418.17	1,675.88
4	P	405.23	1,562.80	501.23	1,562.80	515.23	1,562.80
5	H	542.28	1,465.74	638.28	1,465.74	652.28	1,465.74
6	L	655.37	1,328.69	751.37	1,328.69	765.37	1,328.69
7	S	742.40	1,215.60	838.40	1,215.60	852.40	1,215.60
8	A	813.44	1,128.57	909.44	1,128.57	923.44	1,128.57
9	P	910.49	1,057.53	1,006.49	1,057.53	1,020.49	1,057.53
10	S	997.52	960.48	1,093.52	960.48	1,107.52	960.48
11	Y	1,160.59	873.45	1,256.59	873.45	1,270.59	873.45
12	L	1,273.67	710.38	1,369.67	710.38	1,383.67	710.38
13	A	1,344.71	597.30	1,440.71	597.30	1,454.71	597.30
14	H	1,481.77	526.26	1,577.77	526.26	1,591.77	526.26
15	Q	1,609.82	389.20	1,705.82	389.20	1,719.82	389.20
16	L	1,722.91	261.15	1,818.91	261.15	1,832.91	261.15
17	E		148.06		148.06		148.06

Supplementary Table 5. Oligonucleotides used in this study.

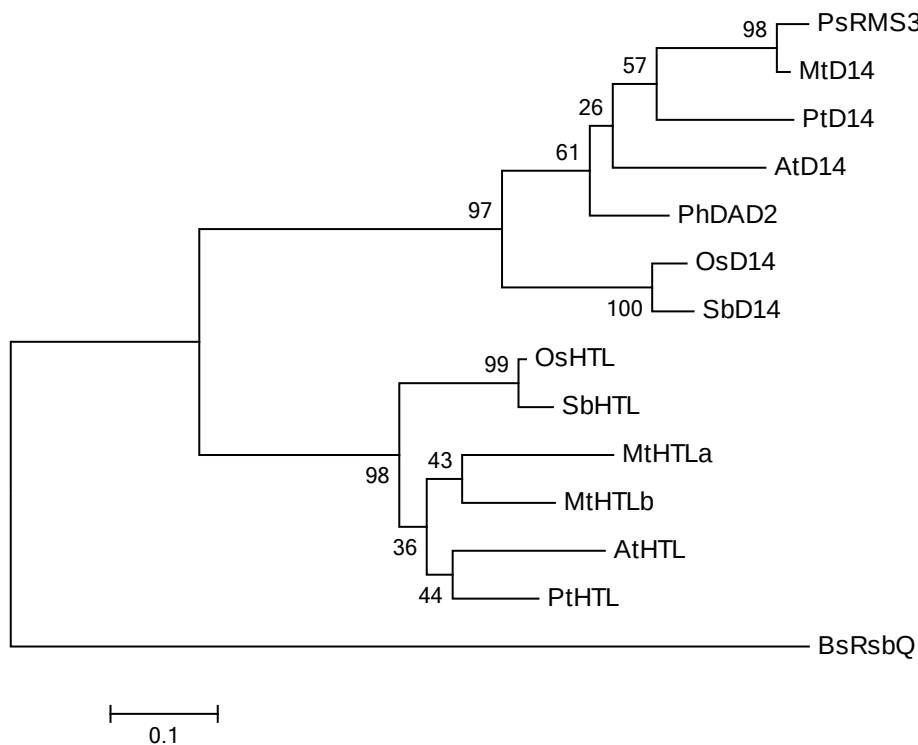
Primer name	Purpose	Sequence (5'→ 3')	Note
Isolation of RMS3 gene and sequencing			
PsD14_138F	degenerate primer	GCACCGACCA(G/A)TCNGCN	
PsD14_701R	degenerate primer	CCGTTTGCAATGATACA(T/C)CANGGNAC	
RMS3_170R	PCR walking	GAAATGATCGGGGTTGACGC	
RMS3_160R	PCR walking	GGGTTGACGCTACCGGCGCA	
RMS3_150R	PCR walking	TACCGGCGCAGACGAGGTCG	
RMS3_741F	3'RACE PCR	CACTATTGCTTTCAAGGACGG	
RMS3_788F	3'RACE PCR	TTAGGGTTGGTTAATGTCCCC	
RMS3_1F	RMS3 sequencing	ATG AAAAACAAAA TCAAACAT	Start codon in bold STOP Codon in bold
RMS3_1312R	RMS3 sequencing	CTA CTGCGAAAGTGCAATCT	
RMS3_142F	RMS3 splicing study	ATCCTCTACGACCTCGTCTG	
RMS3_579R	RMS3 splicing study	CCCTTGCTCAAATCCACCAT	
Cloning for complementation assay			
AtD14_promo_attB4	AtD14 promoter	ggggacaactttgtatagaaaagtgccCCTCTTGTGGATTCTTGGC	attB4 site underlined
AtD14_promo_attB1R	AtD14 promoter	ggggactgctttttgtacaaacttgcTTTTTATGTGTTTGGGTTTGAGG	attB1R site underlined
AtD14_attB1	AtD14 coding sequence	ggggacaagttgtacaaaaagcaggcttc ATG AGTCAACACAAATCTT	attB1 site underlined
AtD14_attB2_ΔS	AtD14 coding sequence	ggggaccactttgtacaagaaagctgggtcCCGAGGAAGAGCTCGCCGGA	attB2 site underlined
Cloning for protein expression			
RMS3_attB1_HRV3	RMS3 coding sequence	ggggacaagttgtacaaaaagcaggctccctggaagtgtgttcagggcccg ATGG GCAC TCCCATCTCTGA	Gene specific sequences are capitalized Start and Stop codons are highlighted in bold
RMS3_attB2	RMS3 coding sequence	ggggaccactttgtacaagaaagctgggtctca CTA CTGCGAAAGTGCAATCT	
AtD14_attB1_HRV3	AtD14 coding sequence	ggggacaagttgtacaaaaagcaggctccctggaagtgtgttcagggcccg ATGA GTCAACACAAATCTT	
AtD14_attB2	AtD14 coding sequence	ggggaccactttgtacaagaaagctgggtctca TCA CCGAGGAAGAGCTCGCC	
AtHTL_attB1_HRV3	AtKAI2 coding sequence	ggggacaagttgtacaaaaagcaggctccctggaagtgtgttcagggcccg ATGG GTGTGGTAGAAGAAAC	
AtHTL_attB2	AtKAI2 coding sequence	ggggaccactttgtacaagaaagctgggtctca TCA CATAGCAATGTCAATTAC	
AtMES9_attB1_HRV3	AtMES9 coding sequence	ggggacaagttgtacaaaaagcaggctccctggaagtgtgttcagggcccg ATGA AGCATATTGTGCTAGT	
AtMES9_attB2	AtMES9 coding sequence	ggggaccactttgtacaagaaagctgggtctca TTAG GGA TATTATCAGCAA	
PCR-Based Mutagenesis			
RMS3 ^{S96A}		CTTACGTAGGTCACGCCATCTCCGCCATG	The underlined codon corresponds to the mutated amino acid
RMS3 ^{S96C}		CTTACGTAGGTCACGCATCTCCGCCATGAC	
RMS3 ^{H247A}		CTTGACACGGAGGGGGCGCTTCCTCATTTGAG	
AtD14 ^{S97A}		CAAAACTGTGCTTACGTTGGTCACGCGGTTTCCGCTATGATCGGAATCA TC	
AtD14 ^{S97C}		CTGTGCTTACGTTGGTCACTGCGTTTCCGCTATGATCGGAATC	
AtD14 ^{H247A}		GACTCTTAAAACTGAAGGAGCGTTGCCGAGCTTAGTGCTC	

Supplementary Table 6. Data collection and refinement statistics.

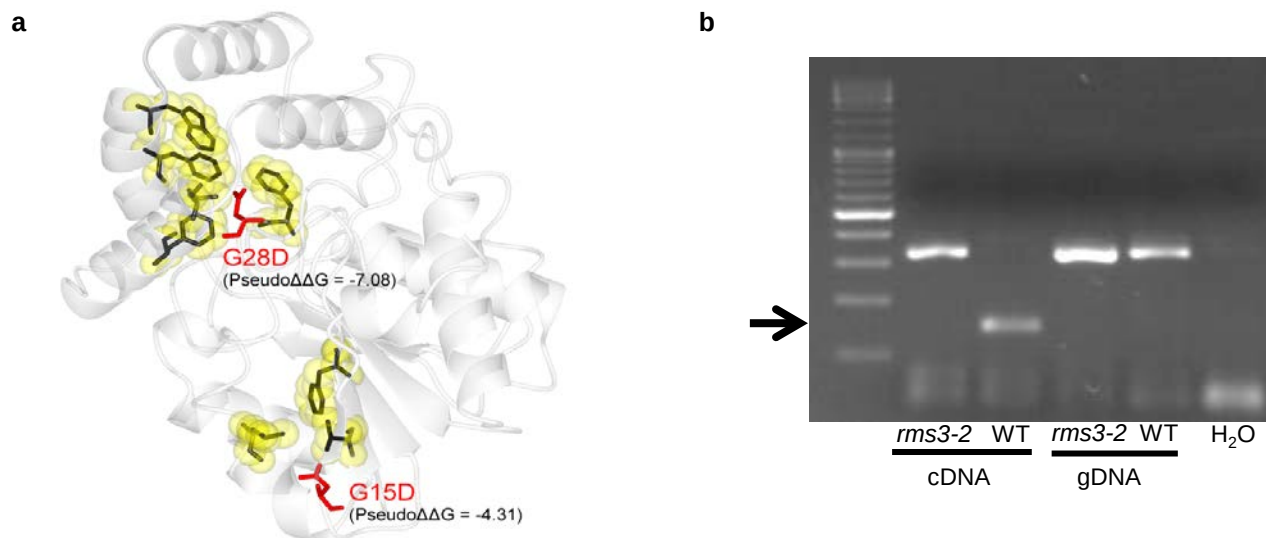
Identification code		(-)-GC242	(+)-GC242
Empirical formula		C ₁₆ H ₁₂ F ₂ O ₅	C ₁₆ H ₁₂ F ₂ O ₅
Formula weight		322.26	322.26
Temperature	(K)	293(2) K	293(2) K
Diffractometer		Rigaku mm007 HF Rotating anode	
Wavelength	(Å)	1.54187	1.54187
Crystal system, Space group		Triclinic, P 1	Triclinic, P 1
Unit cell dimensions	a (Å)	4.0838(4)	4.0850(2)
	b	6.5227(6)	6.5169(4)
	c	13.3729(11)	13.3655(9)
	a (°)	80.870(6)	80.875(6)
	b	86.736(6)	86.742(6)
	g	84.545(6)	84.539(6)
Volume	(Å ³)	349.80(6)	349.40(4)
Z,		1,	1,
Calculated density	(Mg/m ³)	1.530	1.532
Absorption coefficient	(mm ⁻¹)	1.133	1.134
F(000)		166	166
Crystal size	(mm)	0.130 x 0.090 x 0.060	0.250 x 0.130 x 0.050
q range for data collection	(°)	3.350 to 68.201	3.352 to 68.229
Limiting indices		-4 ≤ h ≤ 4, -7 ≤ k ≤ 7, -16 ≤ l ≤ 16	-4 ≤ h ≤ 4, -6 ≤ k ≤ 7, -15 ≤ l ≤ 16
Reflections collected / unique		4746 / 2125	4679 / 2099
R(int)		0.0590	0.0508
Completeness to q = 67.69° (%)		98.1	97.0
Absorption correction		Semi-empirical from equivalents	
Max. and min. transmission		1.00 and 0.68	0.94 and 0.70
Refinement method		Full-matrix least-squares on F ²	
Data / restraints / parameters		2123 / 3 / 212	2099 / 3 / 211
Goodness-of-fit on F ²		1.007	1.070
Final R indices [I > 2σ(I)]	R1	0.0826	0.0401
	wR2	0.1561	0.0904
R indices (all data)	R1	0.1542	0.0635
	wR2	0.1967	0.1151
Absolute structure parameter		0.02(15)	0.02(15)
Extinction coefficient		0.022(6)	n/a
Largest diff. peak and hole (e.Å ⁻³)		0.255 and -0.228	0.172 and -0.237



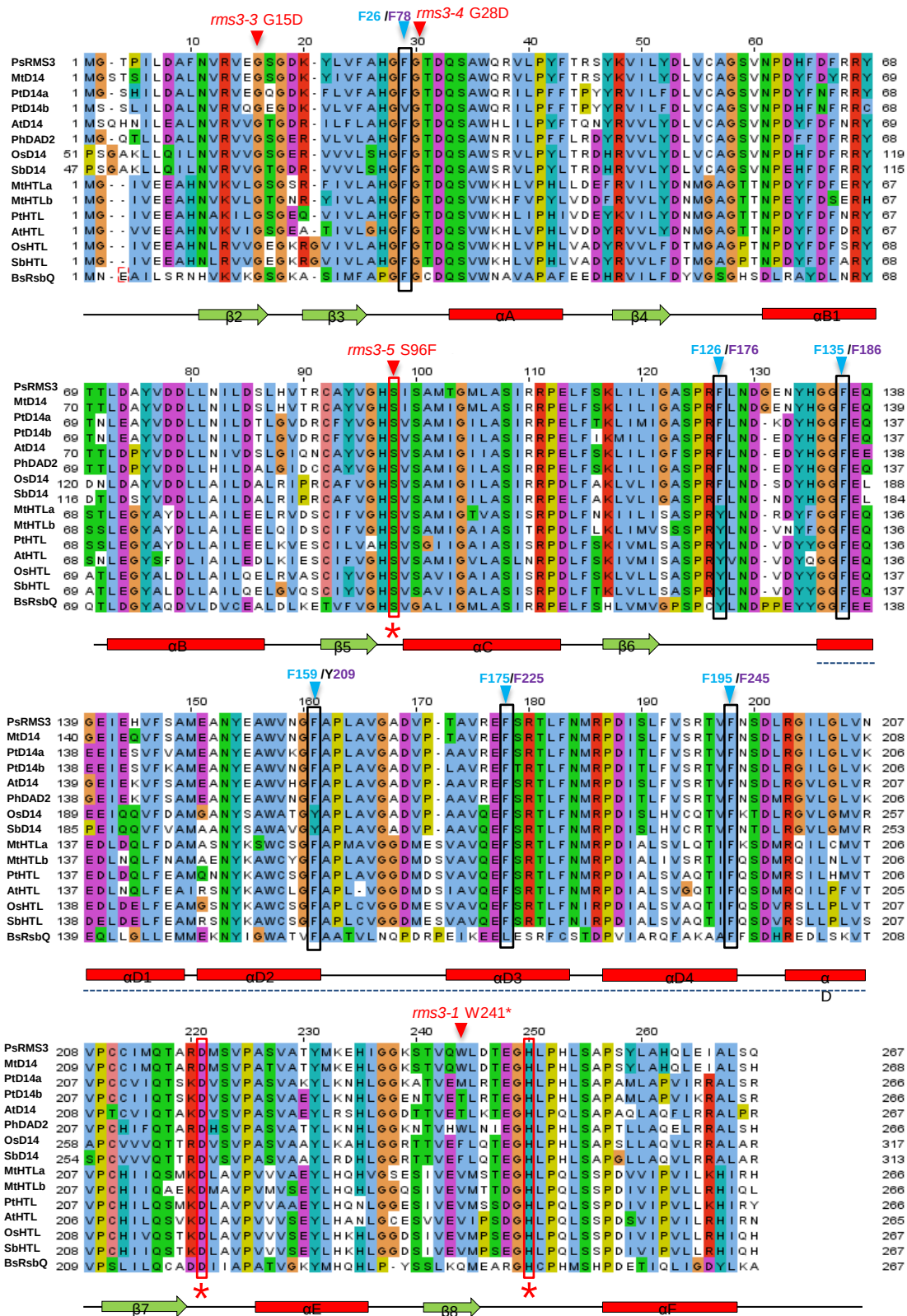
Supplementary Figure 1. RMS3 is the pea homolog of the Arabidopsis AtD14 SL receptor. (a) Phenotype of *rms3-1* (middle) and *rms3-2* (right) pea mutants identified in Torsdag background (left) (21-d-old). (b) Phenotype of *rms3-3* (right) pea mutant identified in Raman background (left) (21-d-old). (c) Phenotypes of *rms3-5* (middle) and *rms3-4* (right) pea mutants identified in Térèse background (left) (21-d-old). Scale bar = 5 cm. (d) Quantification of SLs exuded from roots of wild-type (Térèse), *rms3-4* and *rms3-5* plants. Exudates were collected into water for 30 h from 20-d-old hydroponically grown plants. Data are means $\pm$ SE, based on analyses of three independent pools of 12 plants for each genotype. (e) 5 *rms3* mutants do not respond to ($\pm$)-GR24 application. SL-deficient *rms1* mutant was used as a positive control. Buds at node 3 of 15-d-old plants were treated with 0 or 1 μ M ($\pm$)-GR24 and their growth measured after 7 d. Data are means $\pm$ SE ($n = 24$). The experiments were repeated twice. (f) Structure of the *RMS3* gene showing the locations of the 5 *rms3* mutations. Bases are numbered from the start codon; intron (thin line). CTL 0 = control 0.



Supplementary Figure 2. Phylogenetic tree of D14 and HTL paralogs from different species (see Supplementary Table 2) using the Maximum Likelihood method based on the Dayhoff matrix based model.¹ The tree with the highest log likelihood is shown. Numbers are percent bootstrap values for 1000 replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5.²

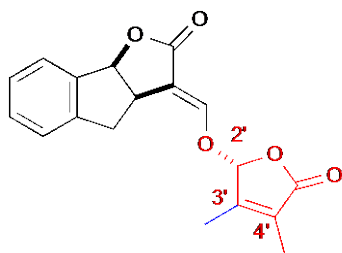


Supplementary Figure 3. Analysis of *rms3* mutations. (a) Location of glycine mutations in *rms3-3* (G15D) and *rms3-4* (G28D) highlighting their potential interference with adjacent hydrophobic pockets. **(b)** Analysis of the nonsplicing of the intron in the *rms3-2* mutant. PCR was performed on leaf cDNA from Torsdag and *rms3-2* with water (H₂O) and genomic DNA (gDNA) as controls. The primer surrounded the intron. Arrow indicates the size expected for the cDNA if the intron is spliced. A 100-bp size marker lane is shown in the left of the gel.



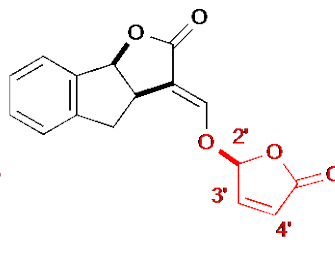
Supplementary Figure 4. Sequence alignment of Pea (Ps) RMS3 protein with D14 and D14-like proteins from Medicago (Mt), Poplar (Pt), Arabidopsis (At), Petunia (Ph), Rice (Os), Sorghum (Sb), and the bacterial RbsQ. Three amino acid residues corresponding to the catalytic triad are marked with asterisks. *rms3* mutations are indicated with a red arrowhead. Essential Phe (F) aromatic residues that probably control the affinity for SL and stabilize the D-ring in the active site gorge of D14 are indicated with a blue arrowhead. Amino acid numbers are indicated for AtD14 (blue) and OsD14 (purple). Note OsD14 protein from rice and SbD14 from Sorghum have a non-conserved 50 amino acid N-terminal extension omitted in the alignment. The C-terminal extension of HTL/D14-like homologs have been omitted in the alignment. The secondary structure assignment is based on the crystal structure of AtD14 and the labels of the major strands and helices are based on Kagiya et al.³ Blue dotted underline comprises the four cap helices.

a



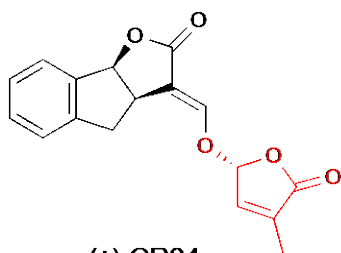
(±)-3'-Me-GR24

LogP = 1.06 ± 0.61



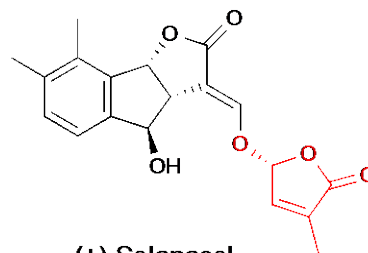
(±)-4'-Desmethyl-2'-*epi*-GR24

LogP = -0.12 ± 0.61



(±)-GR24

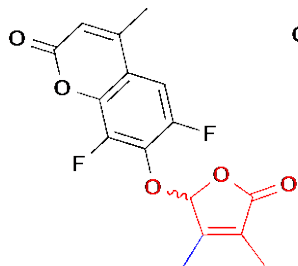
LogP = 0.47 ± 0.61



(±)-Solanaol

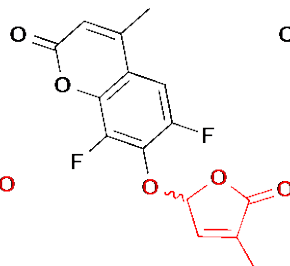
LogP = 0.36 ± 0.64

b



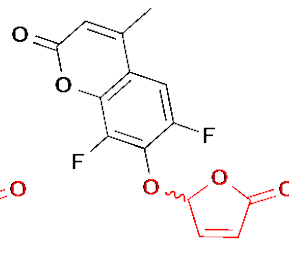
(±)-GC242 **3**

LogP = 1.12 ± 0.77



(±)-GC240 **1**

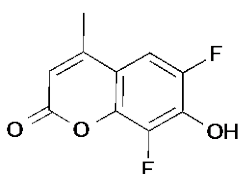
LogP = 0.74 ± 0.72



(±)-GC486 **2**

LogP = 0.15 ± 0.72

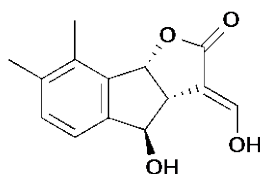
c



DiFMU

LogP = 3.2 ± 0.78

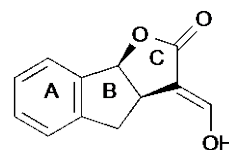
pKa = 6.0 ± 0.9



(±)-7,8-Dimethyl-4-hydroxy-ABC tricycle

LogP = 1.02 ± 0.4

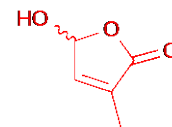
pKa = 5.0 ± 0.5



(±)-ABC tricycle

LogP = 1.1 ± 0.4

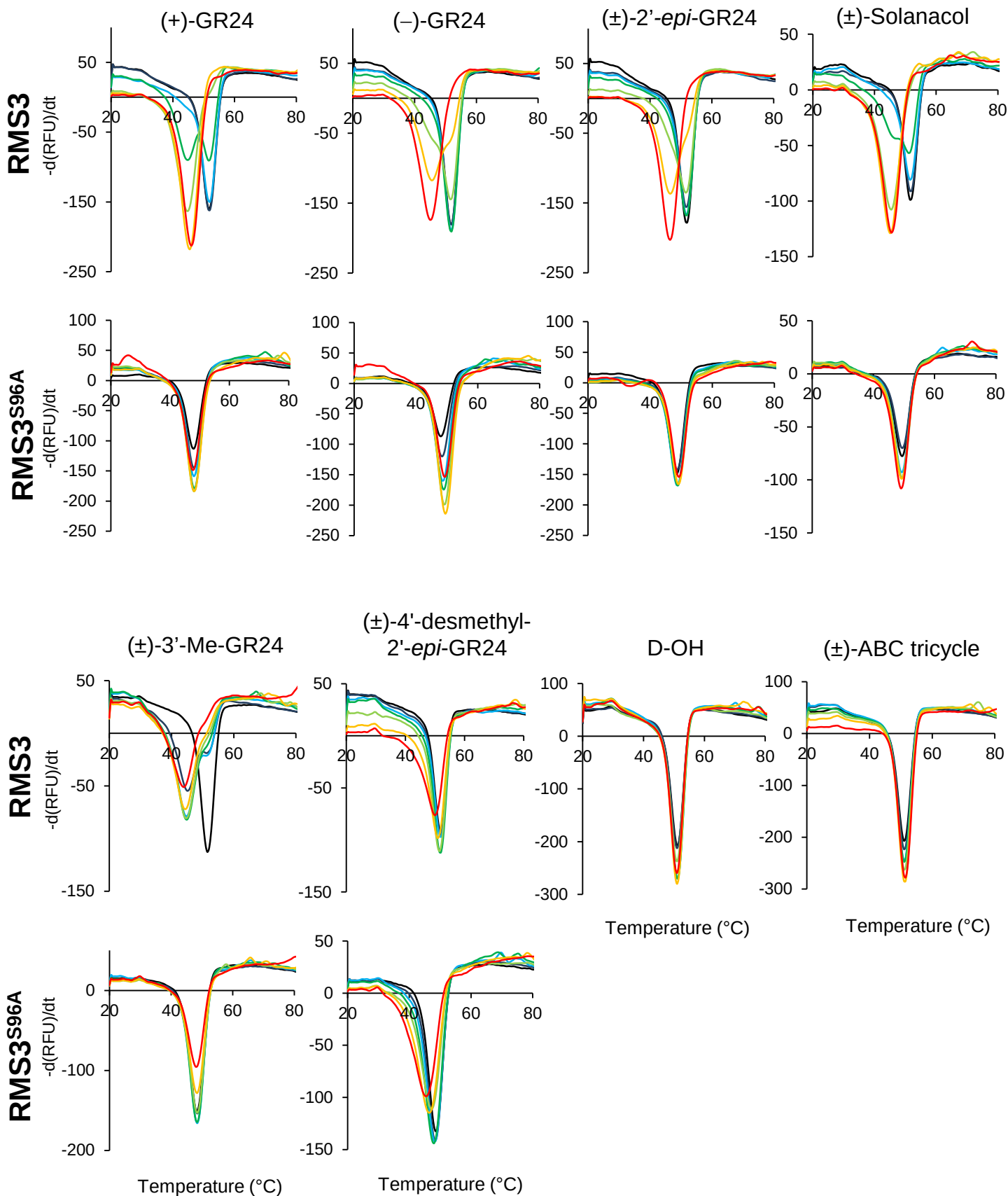
pKa = 5.6 ± 0.4



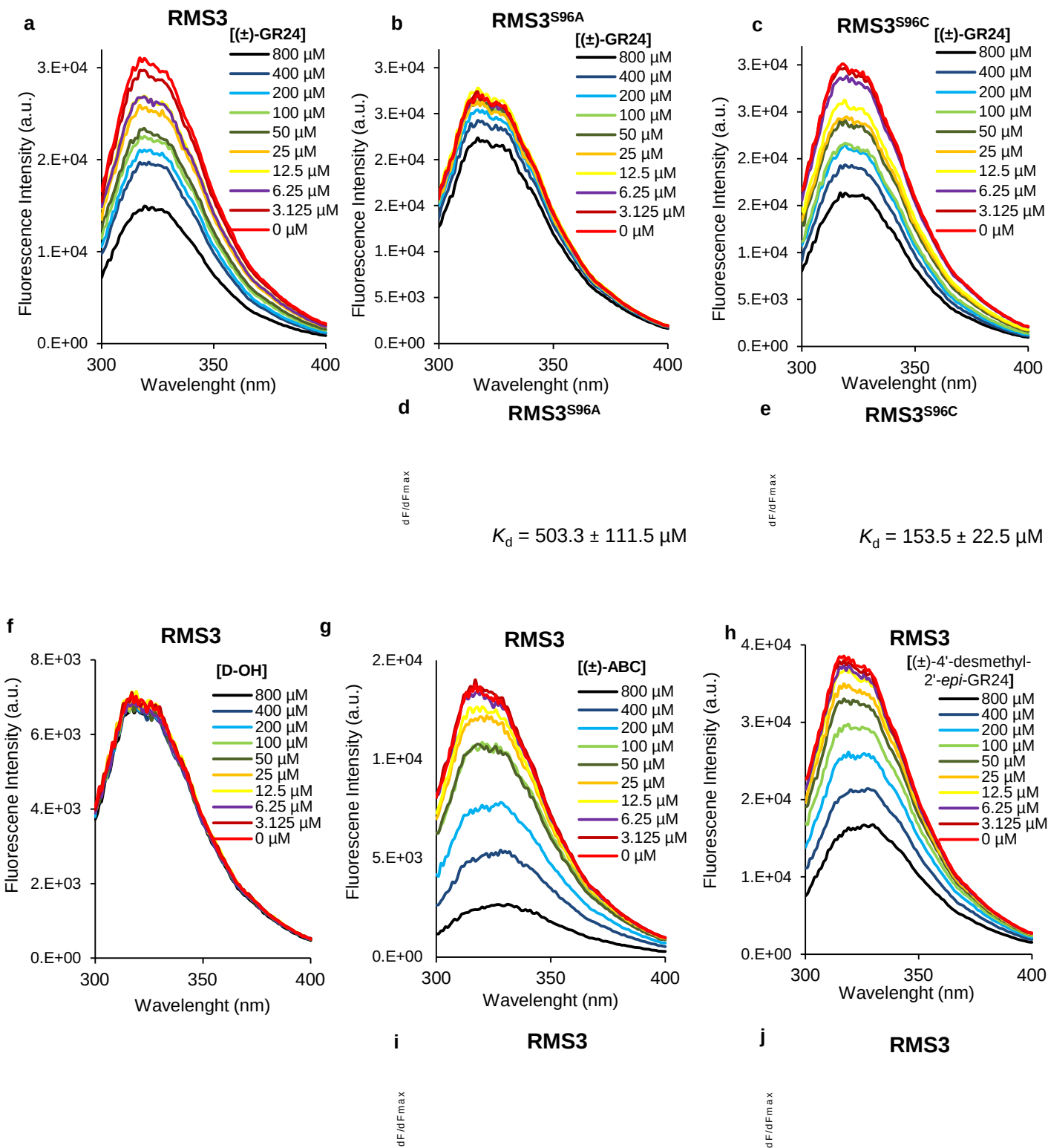
D-OH

LogP = -1.48 ± 0.42

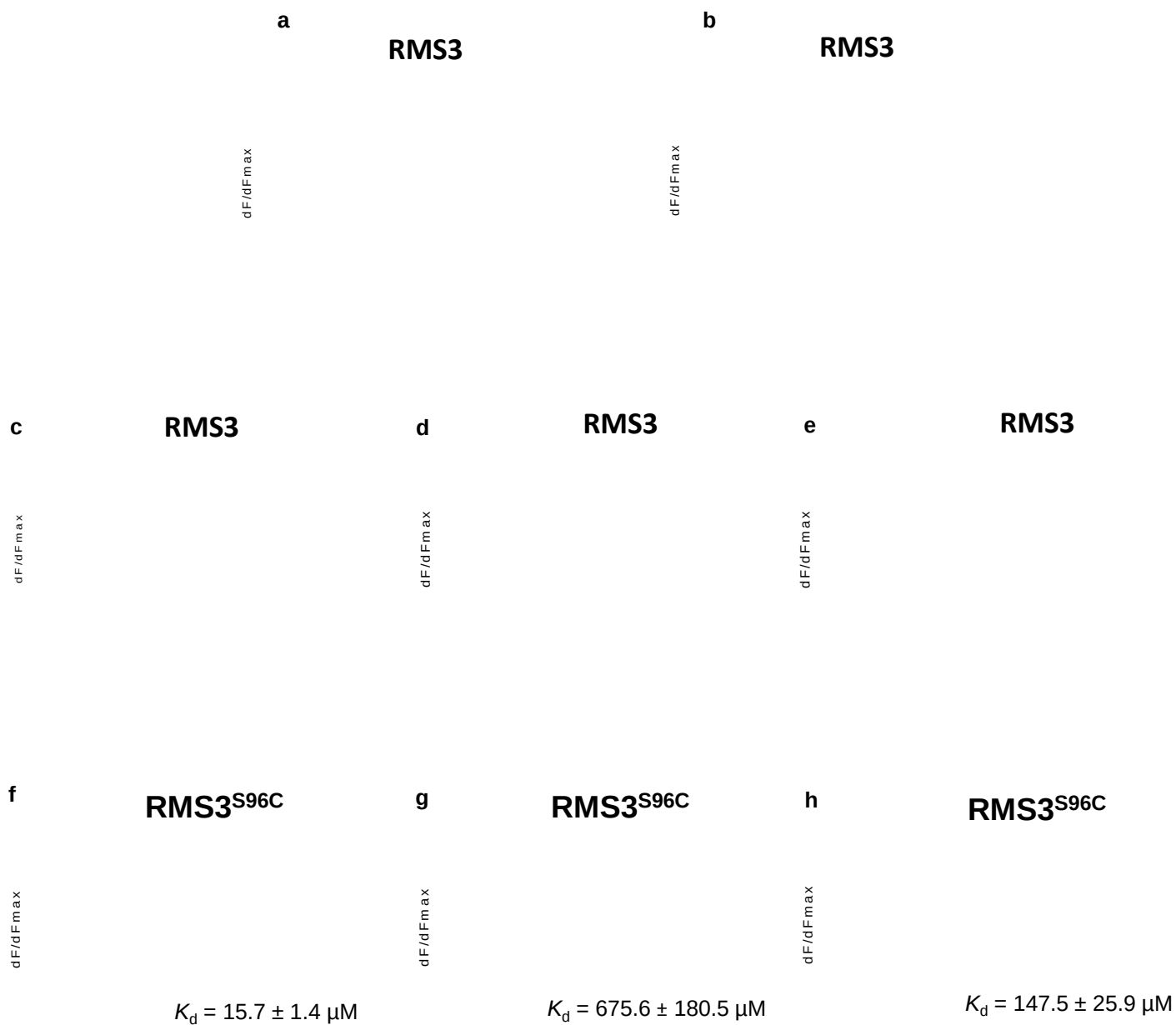
Supplementary Figure 5. Chemical structures of strigolactone analogs and profluorescent probes. (a) Chemical structure of (±)-3'-Me-GR24, (±)-4'-Desmethyl-2'-*epi*-GR24, GR24 and solanaol. (b) Chemical structures of profluorescent probes (±)-GC242, (±)-GC240 and (±)-GC486. (c) Chemical structures of DiFMU, (±)-7,8-Dimethyl-4-hydroxy-ABC tricycle, (±)-ABC tricycle and D-OH. Relative hydrophobicity and pKa Value of SL analogs and probes are indicated for discussion. The LogP, pKa were calculated using the ACD program. (Advanced Chemistry Development, Inc.: <https://ilab.acdlabs.com/ilab2/>)



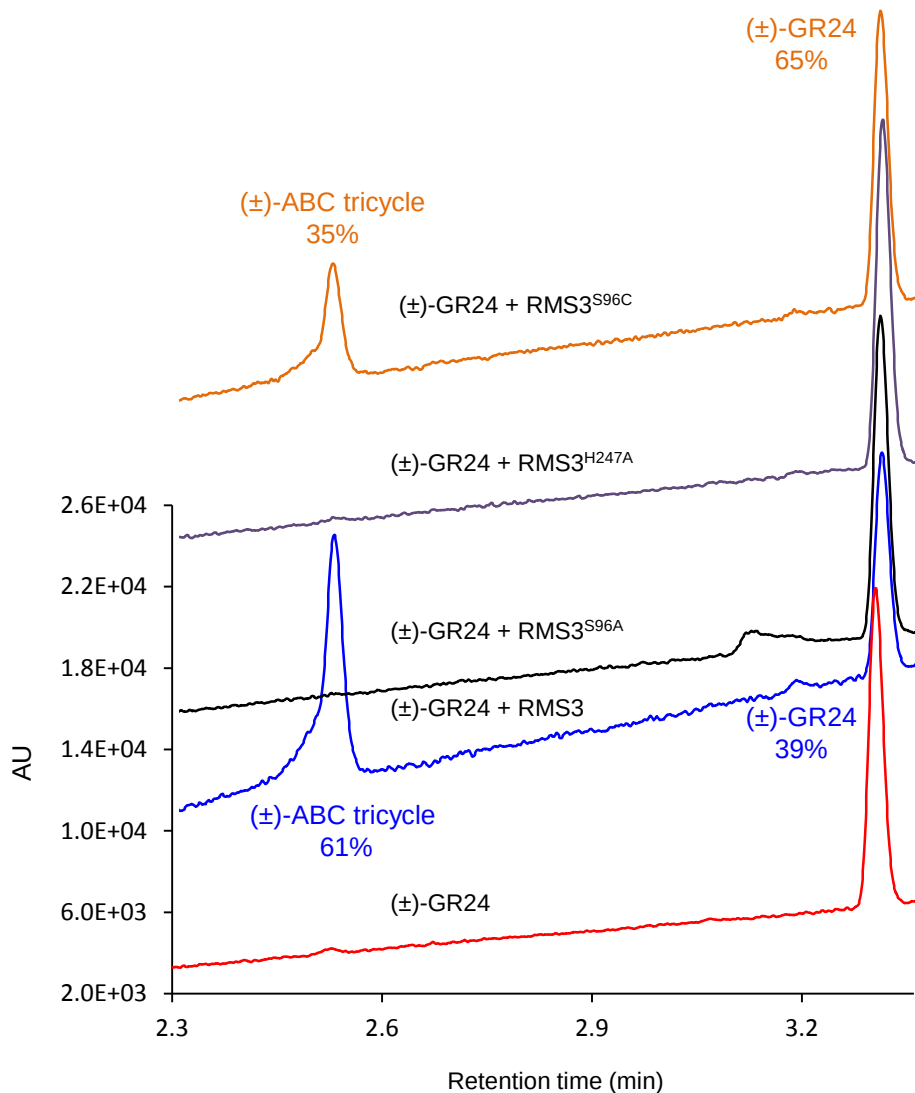
Supplementary Figure 6. Biochemical analysis of the interaction between RMS3 or RMS3^{S96A} and putative ligands as assessed by DSF. Melting temperature curves of RMS3 or RMS3^{S96A} at varying concentrations of (+)-GR24, (-)-GR24, (±)-2'-epi-GR24, (±)-solanacol, (±)-3'-Me-GR24, (±)-4'-desmethyl-2'-epi-GR24, D-OH, (±)-ABC tricycle. Each line represents the average protein melt curve for three replicate samples run in parallel and the experiment was carried out twice with similar results.



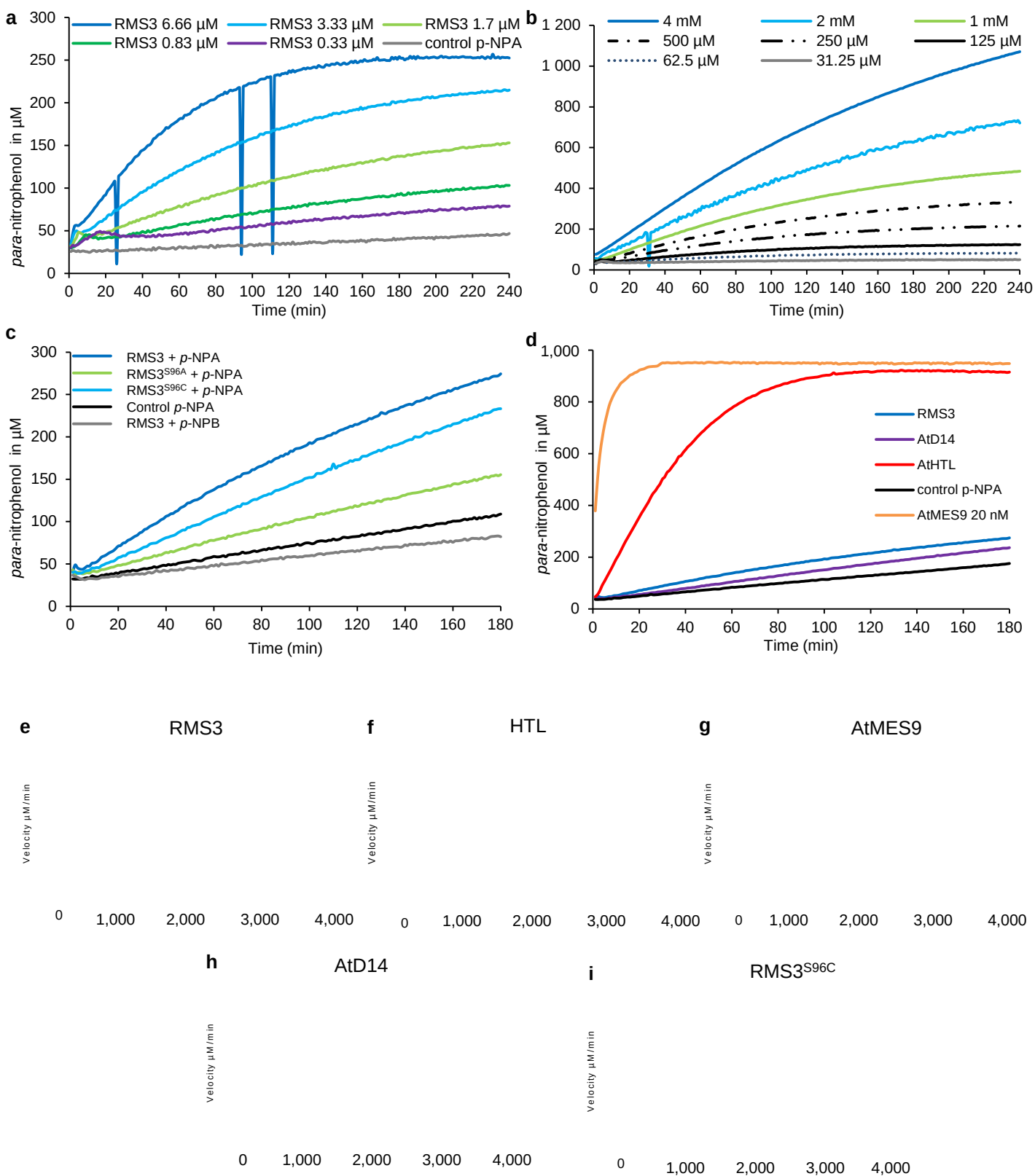
Supplementary Figure 7. Intrinsic tryptophan fluorescence of RMS3 in the presence of SL analogs. (a-c;f-h) Changes in intrinsic fluorescence emission spectra of RMS3, RMS3^{S96A} and RMS3^{S96C} in the presence of various concentrations of (±)-GR24 (a-c), D-OH (f) (±)-ABC (g) or (±)-4'-desmethyl-2'-epi-GR24 (h). Proteins (10 μ M) were incubated with increasing amounts of ligand (0–800 μ M, top line to bottom line, respectively). The observed relative changes in intrinsic fluorescence were plotted as a function of SL analog concentration and transformed to degree of saturation. (d-e; i-j) Plots of fluorescence intensity *versus* (±)-GR24 (d-e) or (±)-ABC (i) or (±)-4'-desmethyl-2'-epi-GR24 (j) concentrations used to determine the apparent K_d values relevant to Table 1. Error bars represent $\pm$ SE of the mean of two replicates and the experiments were repeated at least three times. The analysis was performed with GraphPad Prism 5.0 Software.



Supplementary Figure 8. Intrinsic tryptophan fluorescence of RMS3 and RMS3^{S96C} in the presence of SL analogs. (a-h) Plots of fluorescence intensity *versus* SL analogs concentration. The change in intrinsic fluorescence was monitored and used to determine the apparent K_d values relevant to Table 1. Error bars represent $\pm$ SE of the mean of two replicates. The analysis was performed with GraphPad Prism 5.0 Software. Binding of (+)-GR24 and ($\pm$)-2'-*epi*-GR24 to RMS3^{S96C} was fitted until 400 μM because the binding showed a biphasic curve. The experiments were repeated at least three times.



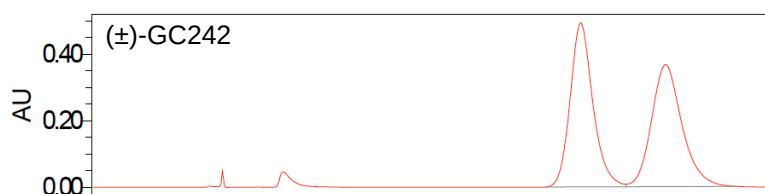
Supplementary Figure 9. Elution profile of the enzymatic assay with buffer, RMS3, RMS3^{S96A}, RMS3^{H247A}, RMS3^{S96C} proteins and (±)-GR24 purified by preparative HPLC. UPLC-UV (254 nm) analysis showing the formation of the (±)-ABC tricyclic (confirmed by mass spectrometry analyses) from (±)-GR24. The indicated percentage corresponds to the relative area of the selected peaks. AU, absorbance unit. The chromatograms show representative results observed in twice independent experiments with two technical replicates.



Supplementary Figure 10. RMS3 hydrolysis activity. (a-d) Progress curves during the 4-nitrophenyl acetate (*p*-NPA) or 4-nitrophenyl butyrate (*p*-NPB) hydrolysis. The release of 4-nitrophenol was monitored (A_{405}) at 25 °C. Solutions contained 100 mM phosphate buffer at pH 6.8. (a) RMS3 catalyzed hydrolysis of *p*-NPA (250 μ M) with different protein concentrations (0.33-6.66 mM). (b) RMS3 (3.33 μ M) catalyzed hydrolysis of *p*-NPA with different substrate concentrations (31.25 mM - 4 mM). (c) Hydrolysis of *p*-NPA (1000 μ M) or *p*-NPB (1000 μ M) by RMS3 and RMS3^{S96A} and RMS3^{S96C} mutant proteins (3.33 μ M). As previously shown with OsD14, *p*-NPB is not hydrolyzed by RMS3 (d) RMS3, KAI2 and AtD14 (3.33 μ M) catalyzed hydrolysis of *p*-NPA (1000 μ M). (e-g) Michaelis-Menten plot of RMS3, RMS3^{S96A}, RMS3^{S96C}, AtD14, HTL and AtMES9 steady state kinetics reaction velocity with *p*-NPA. (h-i) Linear fit of AtD14 and RMS3^{S96C} steady state kinetics reaction velocity with *p*-NPA. Each data point is the mean $\pm$ SD of three technical replicates and the experiments were repeated at least two times.

a

Retention time (min)	Area	% Area
12.935	20,628,962	50.17
15.177	20,487,924	49.83



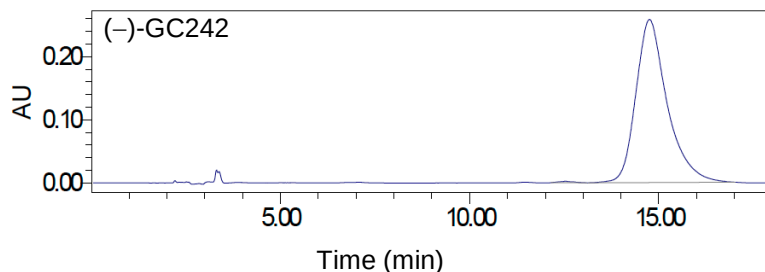
b

Retention time (min)	Area	% Area
13.080	6,338,651	97.50
15.308	162,734	2.50



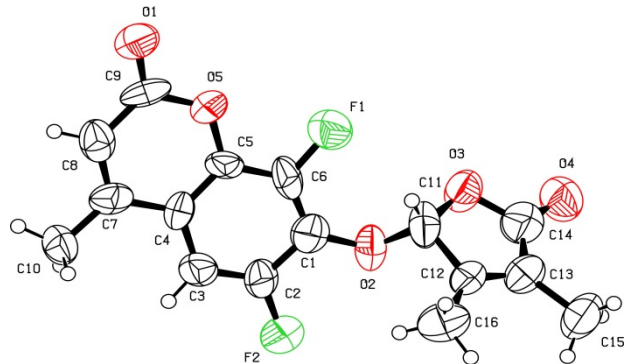
c

Retention time (min)	Area	% Area
12.542	5,570	0.37
14.754	14,838,235	99.63

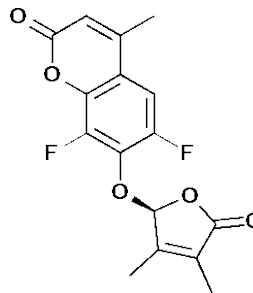
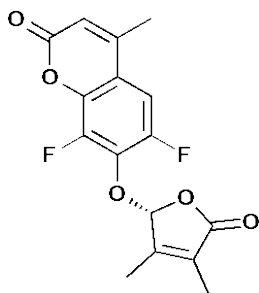
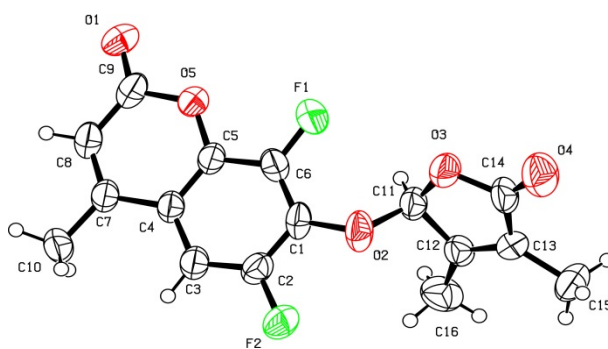


d

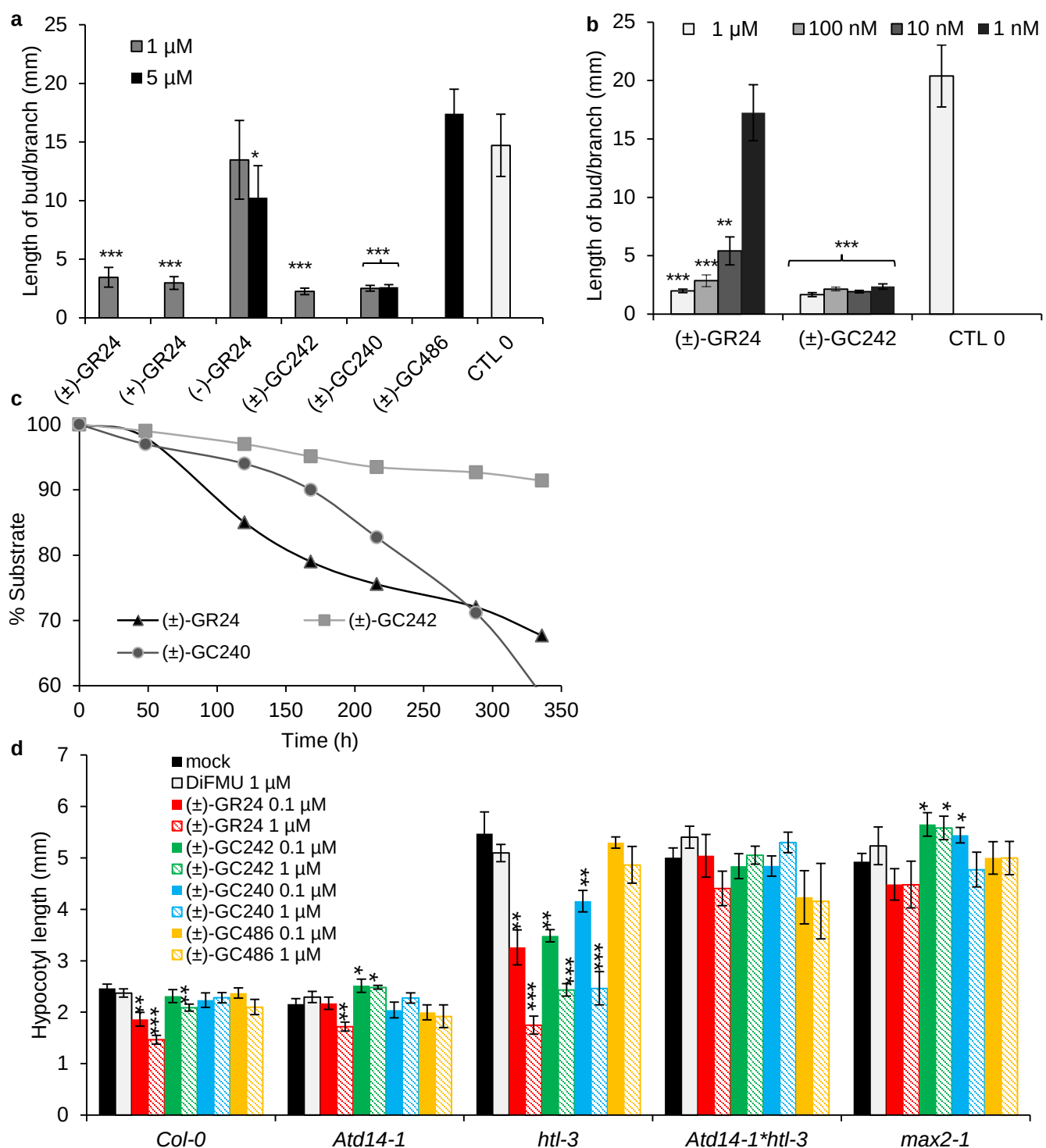
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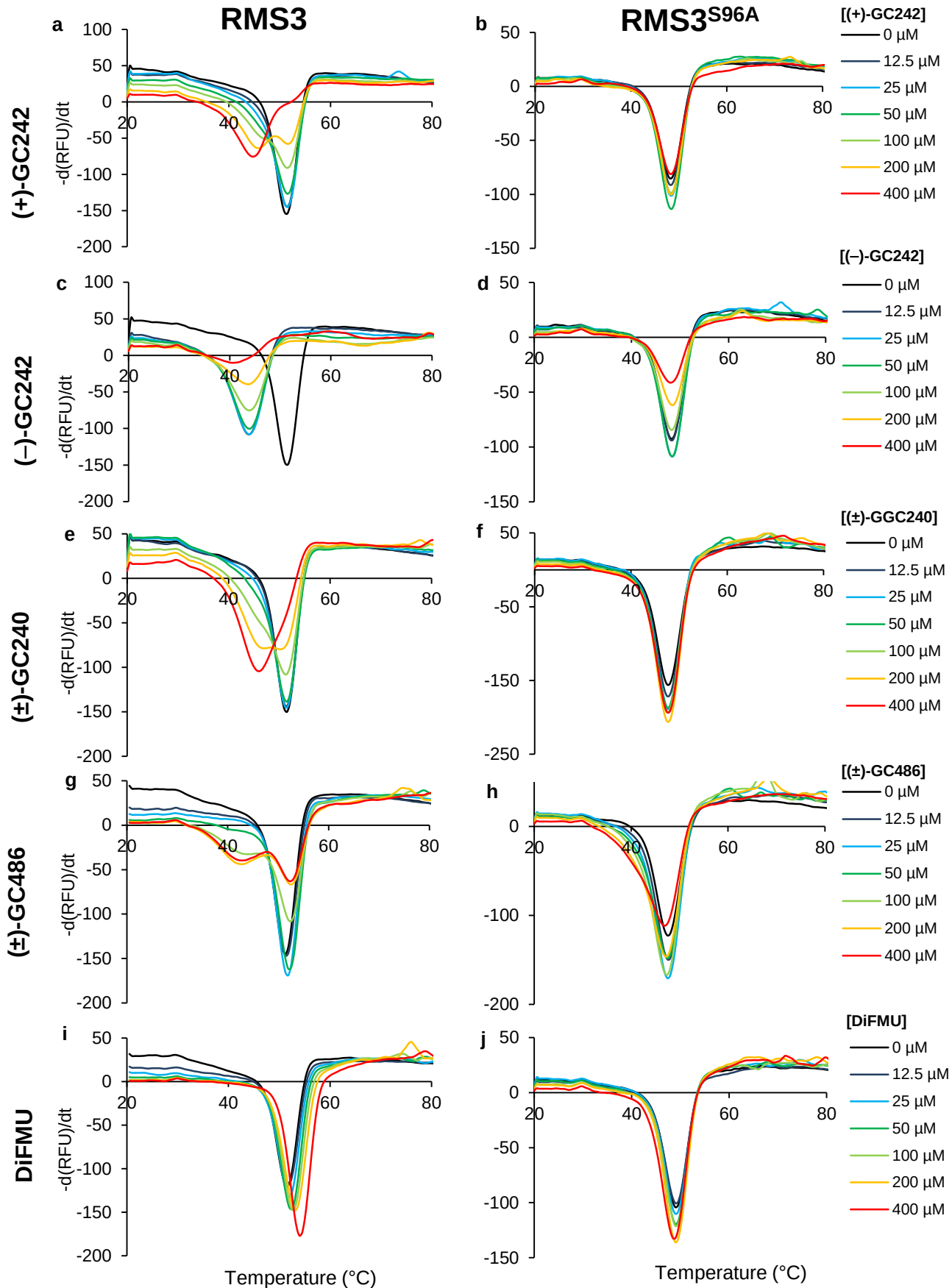
(+)-(S)-GC242



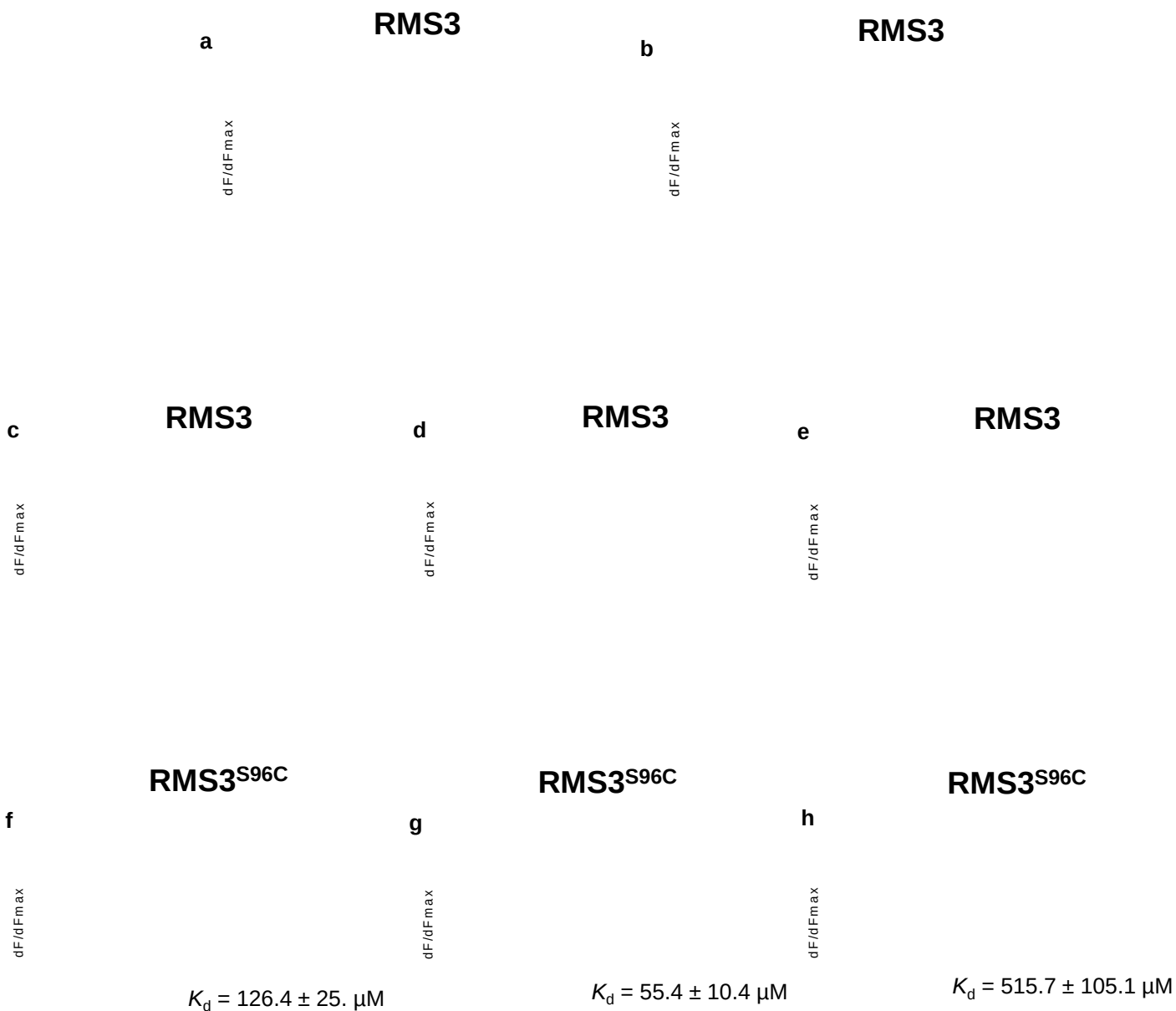
Supplementary Figure 11. Characterization of GC242 enantiomers. (a) Chiral LC-separation of GC242 enantiomers; chromatogram of (±)-GC242 (UV (274 nm) analysis). (b) Chromatogram of (+)-GC242 (UV (274 nm) analysis). (c) Chromatogram of (-)-GC242 (UV (274 nm) analysis). (d) X-ray crystal structure of both enantiomers of GC242. (-)-GC242 is the slow moving and (+)-GC242 the fast moving enantiomer. Atomic displacement parameters are drawn at the 50% probability level. AU, Absorbance Unit.



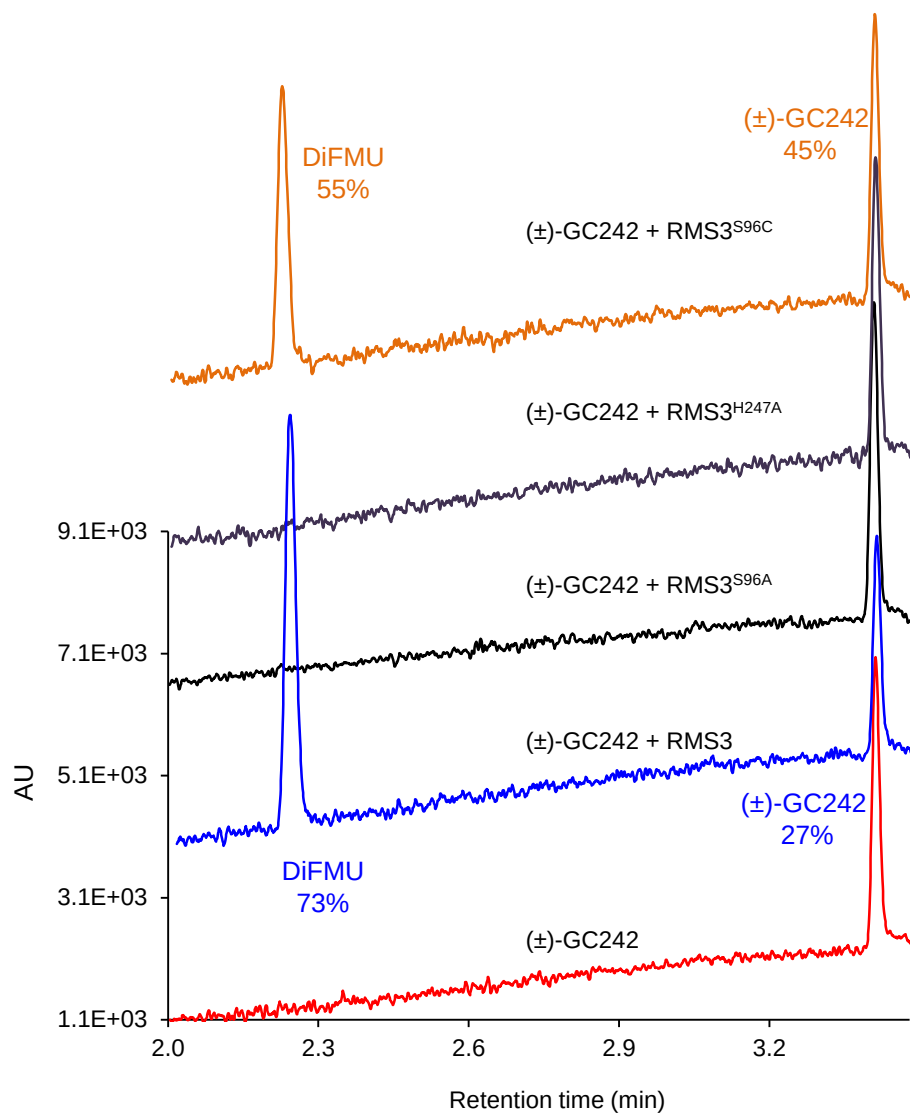
Supplementary Figure 12. Test of ligands and probes bioactivity in pea and *Arabidopsis* and chemical stability of profluorescent probes. (a) Length of axillary bud of *rms1* pea plants, 8 days after direct application of profluorescent probes ($\pm$)-GC242, ($\pm$)-GC240, ($\pm$)-GC486, and ($\pm$)-GR24/(+)-GR24/(-)-GR24 at 5 and/or 1 μM . Data are means $\pm$ SE of at least 16 plants. Asterisks indicate significant differences from control values ($***p < 0.001$, $**p < 0.01$, Kruskal-Wallis rank sum test). (b) Length of axillary bud of *rms1* pea plants, 8 days after direct application of a SL analogue solution at 1 μM , 100, 100, 10 and 1 nM at node 3 ($\pm$)-GR24, ($\pm$)-GC242). Data are means $\pm$ SE of at least 17 plants. Asterisks indicate significant differences from control values ($***p < 0.001$, $**p < 0.01$, Kruskal-Wallis rank sum test). The experiments were repeated at least twice. (c) Chemical stability of ($\pm$)-GR24, ($\pm$)-GC240, ($\pm$)-GC242 in a DMF:PBS (1:1, v/v) (pH 6.8) solution at 21°C. (d) *Arabidopsis* hypocotyl length in response to ($\pm$)-GR24, ($\pm$)-GC242, ($\pm$)-GC240, ($\pm$)-GC486 at 0.1 μM and 1 μM or DiFMU at 1 μM , of Col-0 (WT), *Atd14-1*, *htl-3*, *Atd14-1 htl-3*, and *max2-1* *Arabidopsis* mutants. Data are means $\pm$ SE of at least 10 plants. Asterisks indicate significant difference from corresponding acetone treatment (CTL) ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$, Student's t test). The experiments were repeated twice.



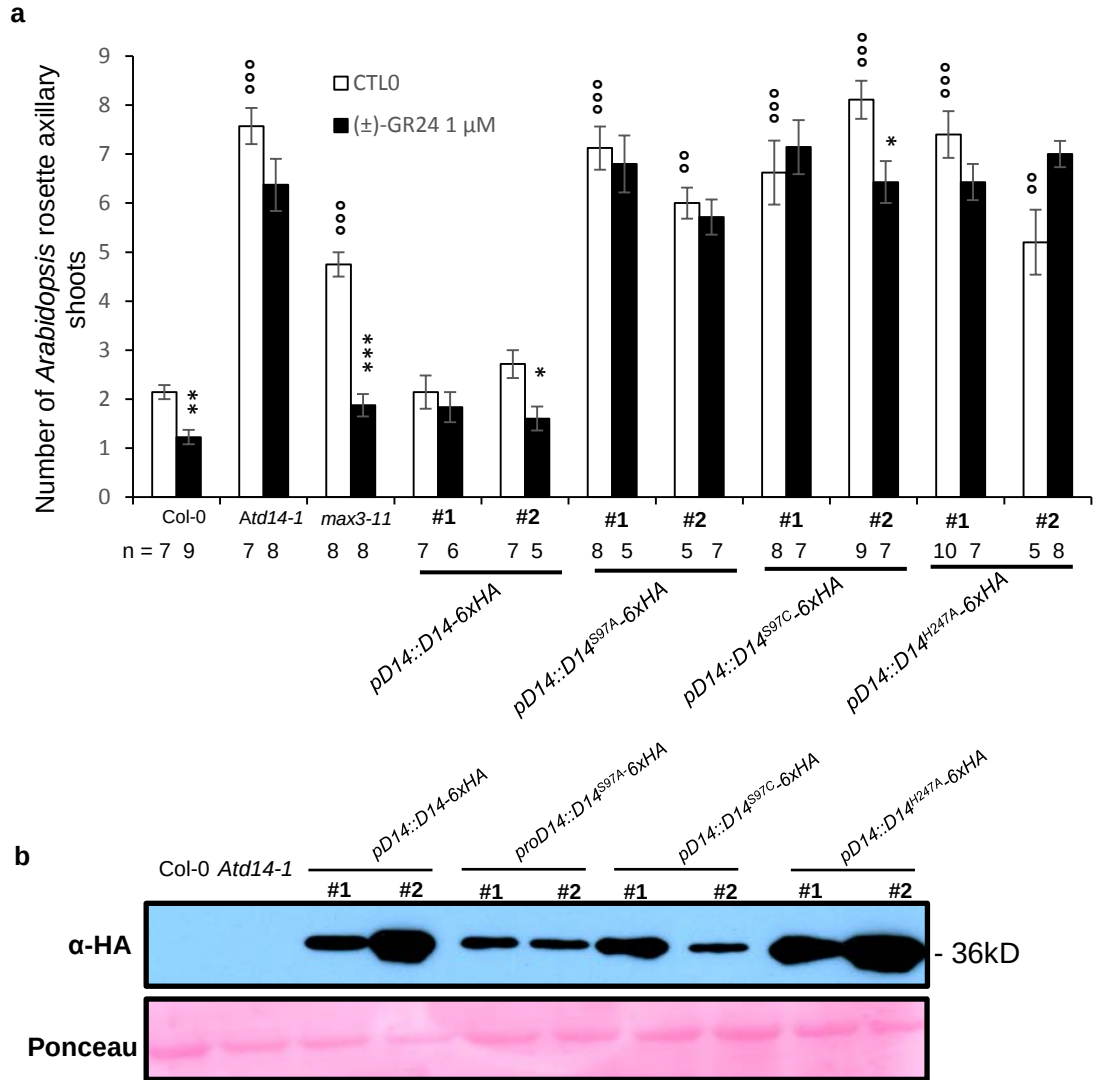
Supplementary Figure 13. Biochemical analysis of the interaction between RMS3 and profluorescent probes as assessed by DSF. (a-j) The melting temperature curves of RMS3 and RMS3^{S96A} with putative probes [(±)-GC240, (+)-GC242, (-)-GC242, (±)-GC486, DiFMU] at varying concentrations are shown as assessed by DSF. Each line represents the average protein melt curve for three technical replicates and the experiment was carried out twice.



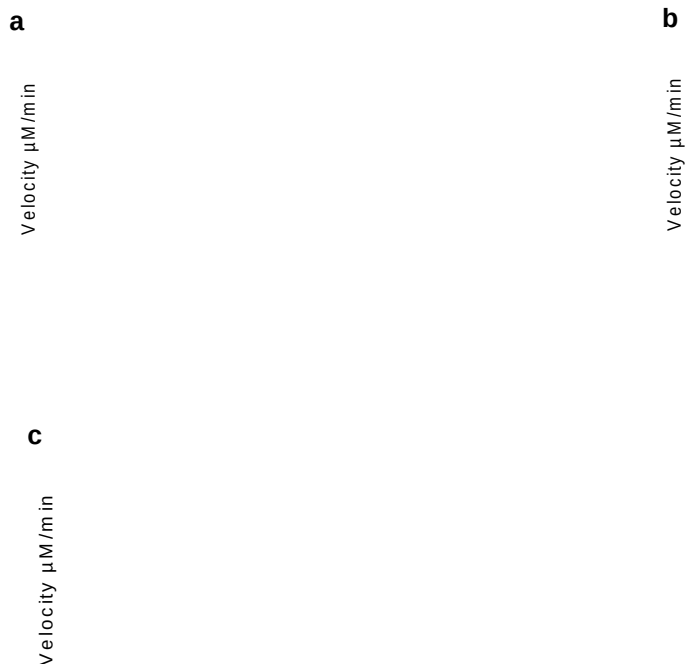
Supplementary Figure 14. Profluorescent probes bind RMS3 and RMS3^{S96C} based on intrinsic tryptophan fluorescence. (a-h) Plots of fluorescence intensity *versus* probe concentrations. The change in intrinsic fluorescence was monitored and used to determine the apparent K_d values relevant to Table 1. Error bars represent $\pm$ SE of the mean of two replicates and the experiments were repeated at least three times. The analysis was performed with GraphPad Prism 5.0 Software. Binding of ($\pm$)-GC242 and (-)-GC242 to RMS3^{S96C} was fitted until 400 μM because the binding showed a biphasic curve.



Supplementary Figure 15. Elution profile of the enzymatic assay with buffer, RMS3, RMS3^{S96A}, RMS3^{H247A}, RMS3^{S96C} proteins and (±)-GC242. UPLC-UV (320 nm) analysis showing the formation of DiFMU (all confirmed by mass spectrometry analyses) from (±)-GC242. The indicated percentage corresponds to the relative area of the selected peaks. AU, absorbance unit. The chromatograms show representative results observed in twice independent experiments with two technical replicates.



Supplementary Figure 16. The catalytic serine and histidine are required for endogenous SLs perception by D14 to inhibit shoot branching. (a) Total number of rosette axillary shoots in T3 40-day-old Arabidopsis plants grown under hydroponics supplemented with 0.1% acetone (CTL0) or 1 μ M ($\pm$)-GR24. Data are means $\pm$ SE (n = number of plants analyzed). Circles indicate significant difference from WT Col-0 ($^{\circ\circ\circ} p < 0.001$, $^{\circ\circ} p < 0.01$, $^{\circ} p < 0.05$, Kruskal-Wallis rank sum test). Asterisks indicate significant difference from corresponding acetone treatment (CTL0) ($^{***} p < 0.001$, $^{**} p < 0.01$, $^{*} p < 0.05$, Kruskal-Wallis rank sum test), CTL0 = control 0. The experiments were repeated twice. (b) D14-6xHA level analyzed by immunoblot using α -HA antibody in Col-0 (wild type), *Atd14-1* and transformed T2 *Atd14-1* plants. Protein extracts from 40-d-old plant leaf were separated by 10% SDS-PAGE and identified as a 36 kD band. Ponceau staining is included for loading reference.



Supplementary Figure 17. Comparison of RMS3 pre-steady state kinetic with different profluorescent probes. (a-d) Hyperbolic plot of the RMS3 presteady state kinetics reaction velocity with (a) ($\pm$)-GC240, (b) (+)-GC242, (c)(–)-GC242. Initial velocity was determined with profluorescent probes concentration from 31.25 nM to 4 μM and RMS3 protein at 400 nM. Error bars represent SE of the mean of three replicates and the experiments were repeated at least three times.

a

Velocity $\mu\text{M}/\text{min}$

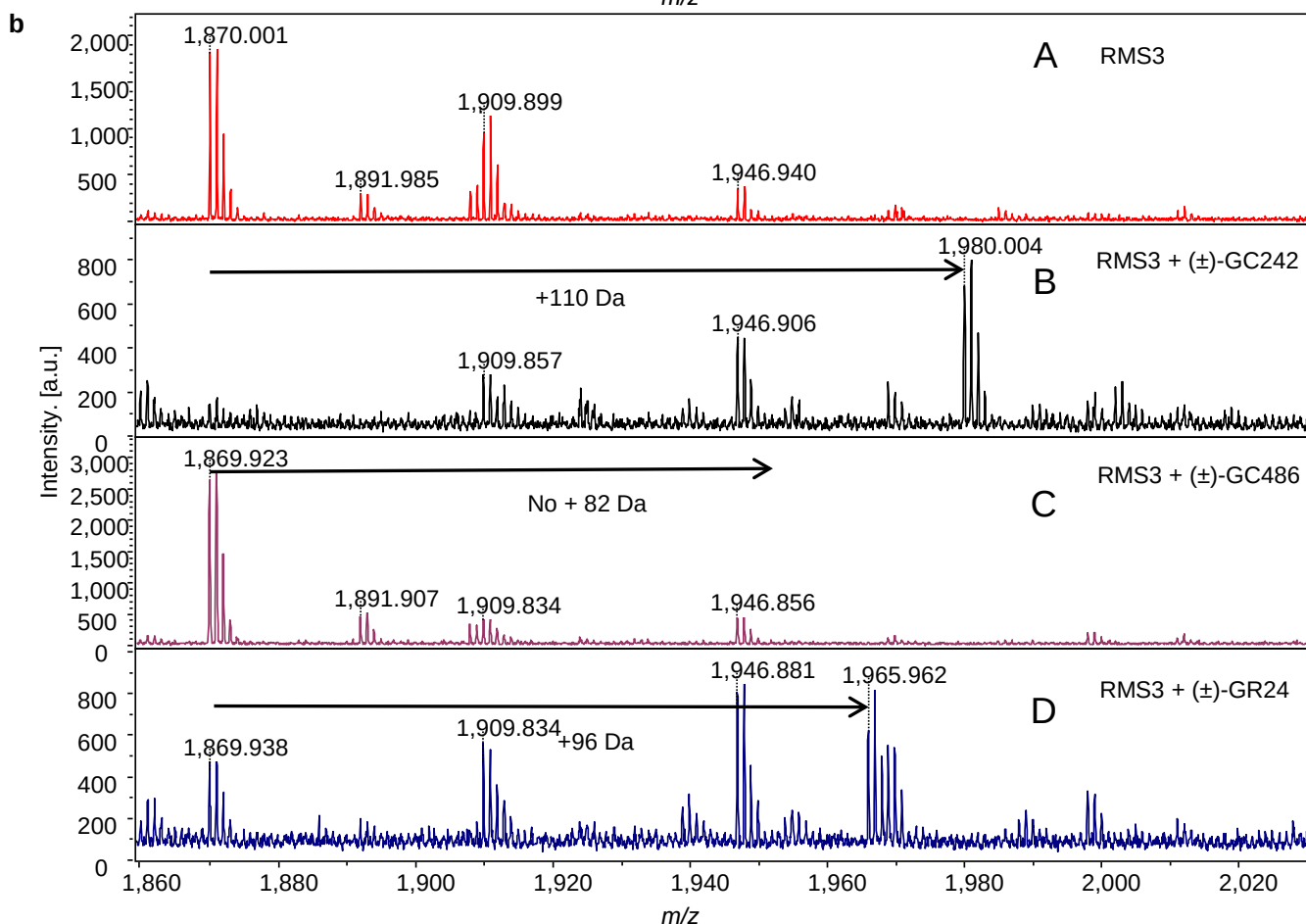
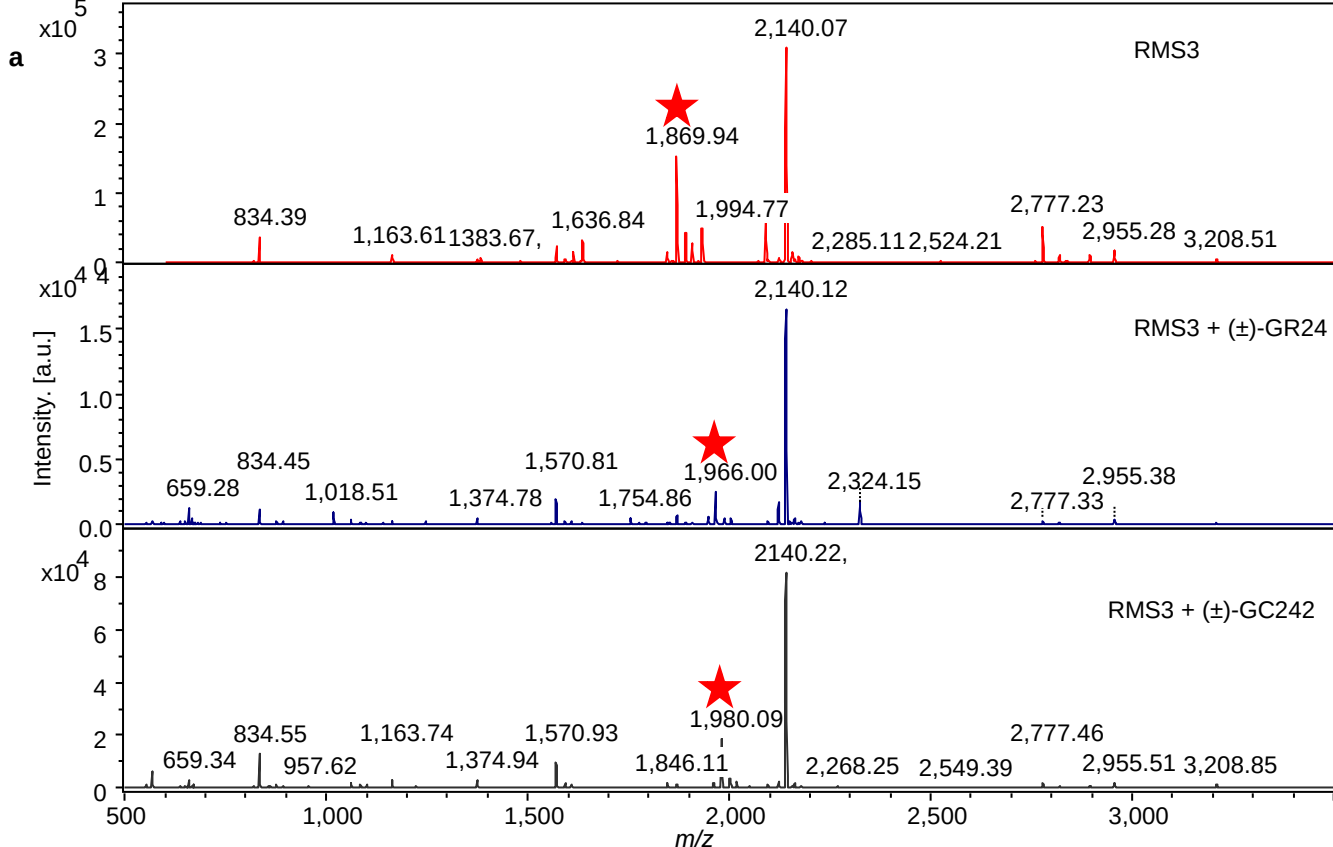
b

Velocity $\mu\text{M}/\text{min}$

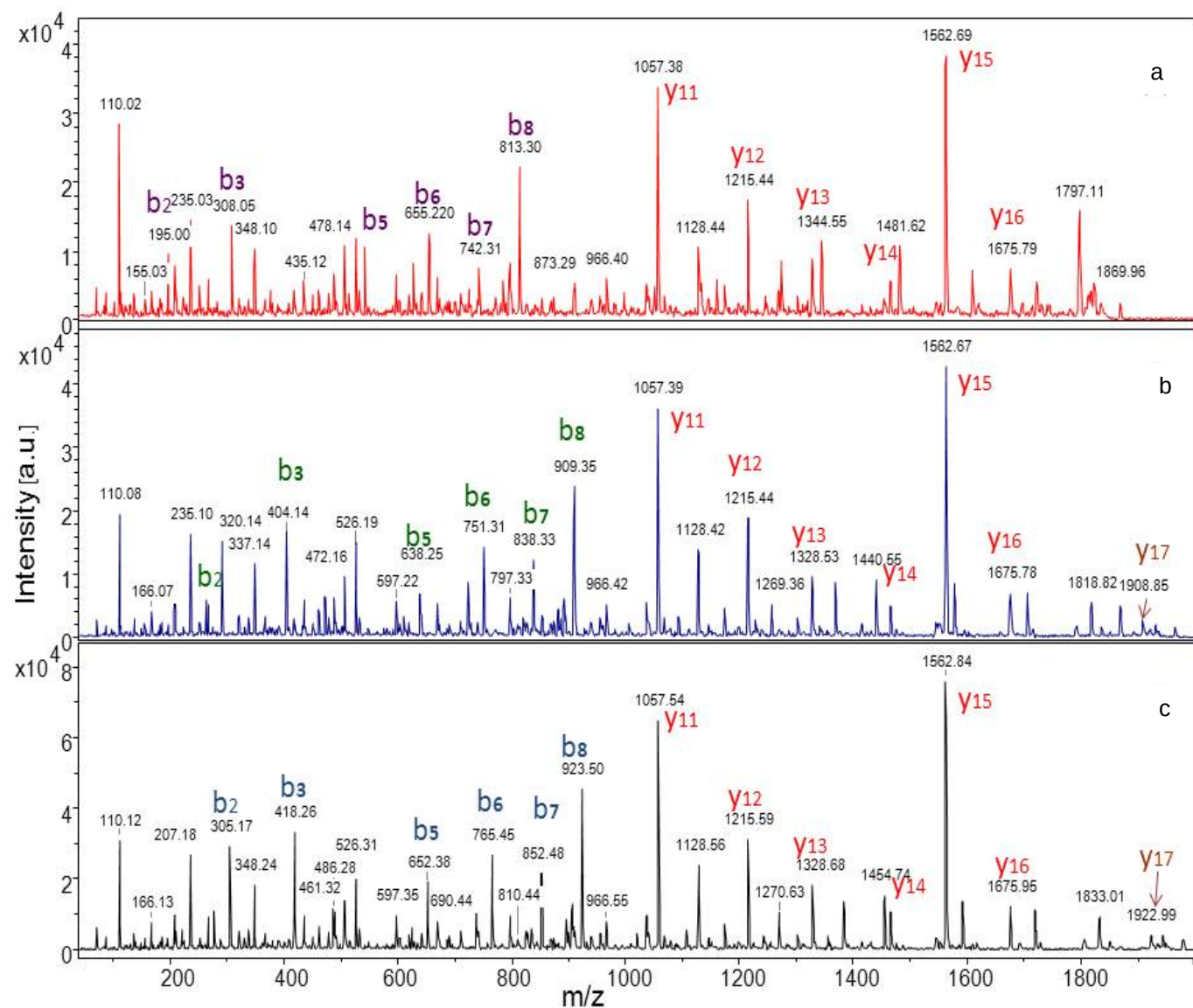
Supplementary Figure 18. Competition assays with (±)-ABC tricycle and (±)-GR24. (a) Effect of (±)-ABC tricycle on the enzyme kinetics of RMS3 shown as a hyperbolic plot. **(b)** Effect of (±)-GR24 on the enzyme kinetics of RMS3 shown as a hyperbolic plot. **(a-b)** Each data point is the mean $\pm$ SE of three technical replicates.



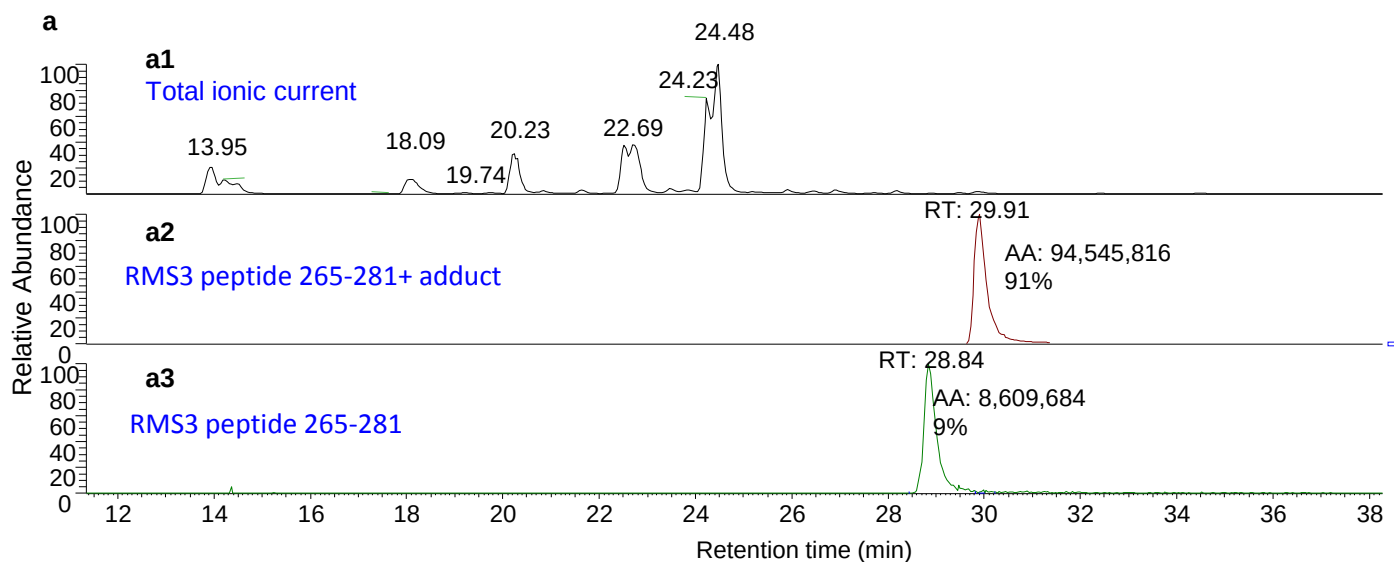
Supplementary Figure 19. Comparison of RMS3 pre-steady state kinetic with ($\pm$)-GC242 and different SL stereoisomers as competitor. (a, b, c, d) Michaelis-Menten plot of the RMS3 presteady state kinetics reaction velocity with ($\pm$)-GC242 (from 31.25 nM to 4 μM) in competition with different SL analog concentrations (from 0,1 μM to 100 μM). These plots were used to determine the K_i values relevant to Table 1 and all calculated K_i values are given in the plots. Error bars represent SE of the mean of three replicates and the experiments were repeated at least two times. The analysis was performed with GraphPad Prism 5.0 Software. The release of DiFMU was monitored (λ_{em} 460 nm) at 25 °C. Solutions contained 100 mM phosphate buffer, pH 6.8 and RMS3 (400 nM).



Supplementary Figure 20. Characterization of the RMS3-D complex. (a) MS spectrum of the mixture of peptides after endoproteinase GluC cleavage. (b) MS spectrum part (m/z 1,860-2,020) of the mixture of peptides after endoproteinase GluC cleavage. A: native peptide, B: complex with ($\pm$)-GC242, C: peptide + ($\pm$)-GC486, D: complex with ($\pm$)-GR24. [a.u.] = arbitrary unit.

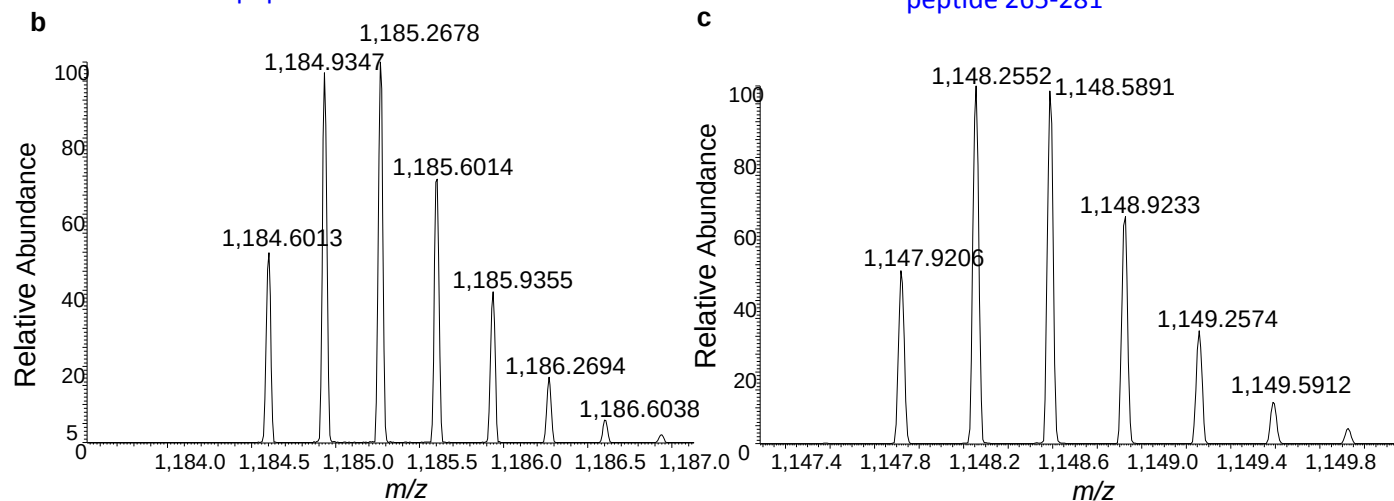


Supplementary Figure 21. Characterization of the RMS3-D complex. Collision-induced dissociation spectrum of peptide 266-282 obtained after endoproteinase GluC cleavage. (a) native peptide, (b) complex with (±)-GR24, (c) complex with (±)-GC242. [a.u.] = arbitrary unit.

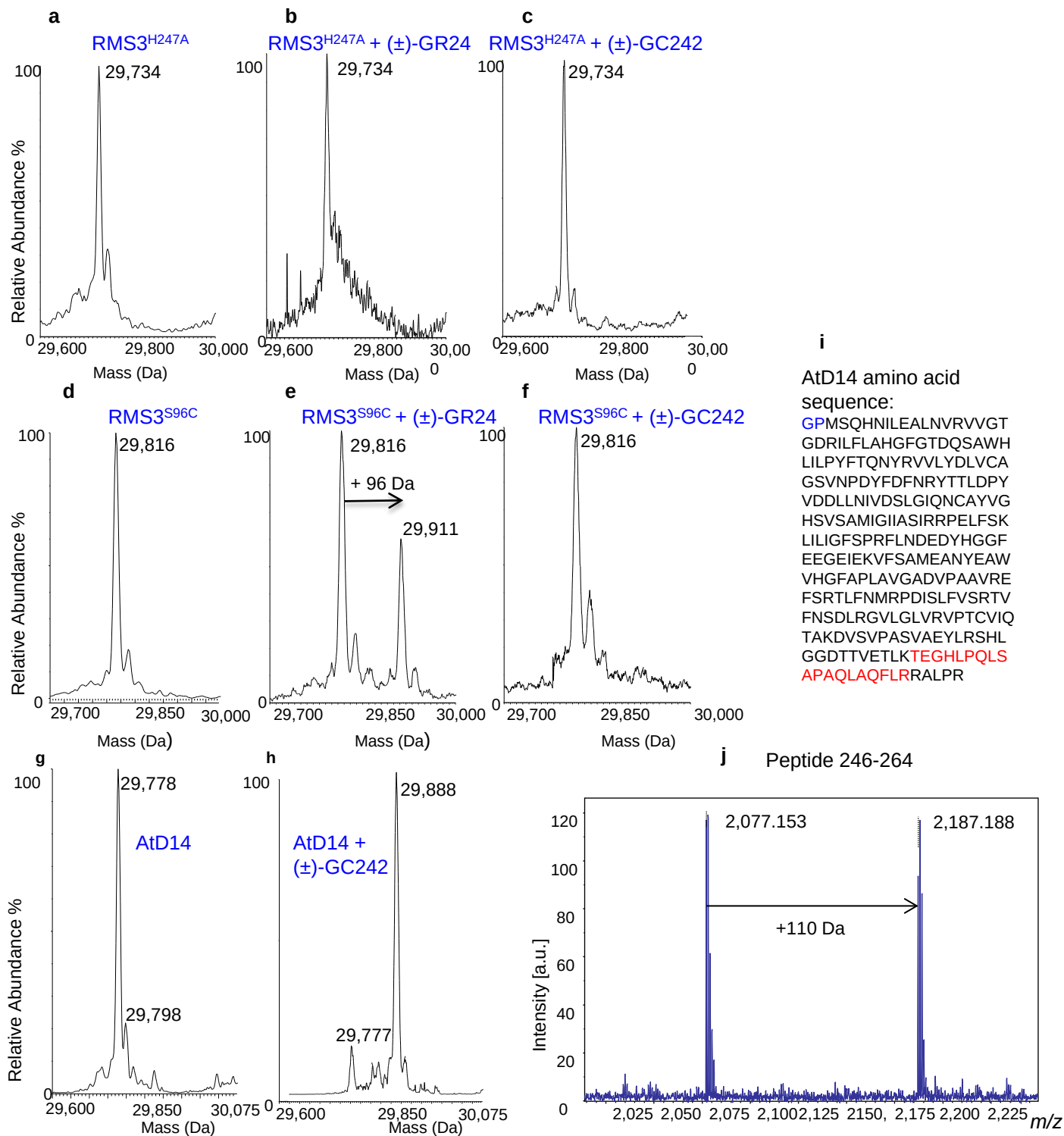


RMS3 peptide 265-281 + adduct

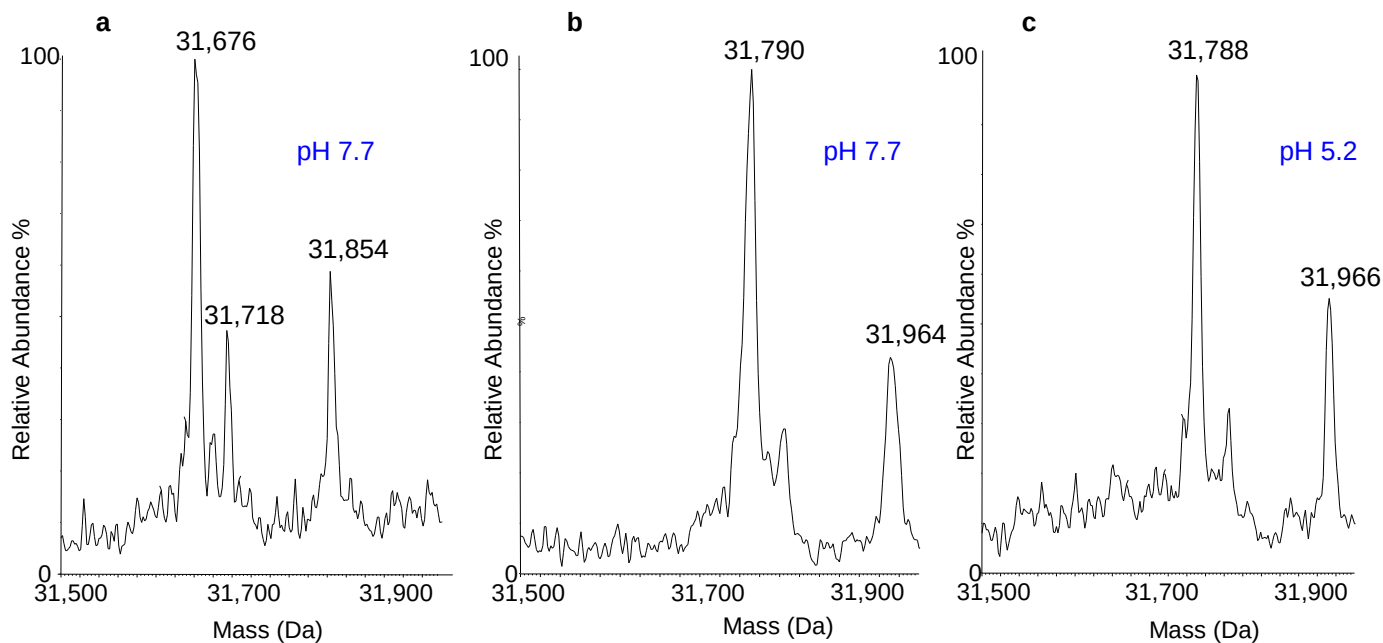
peptide 265-281



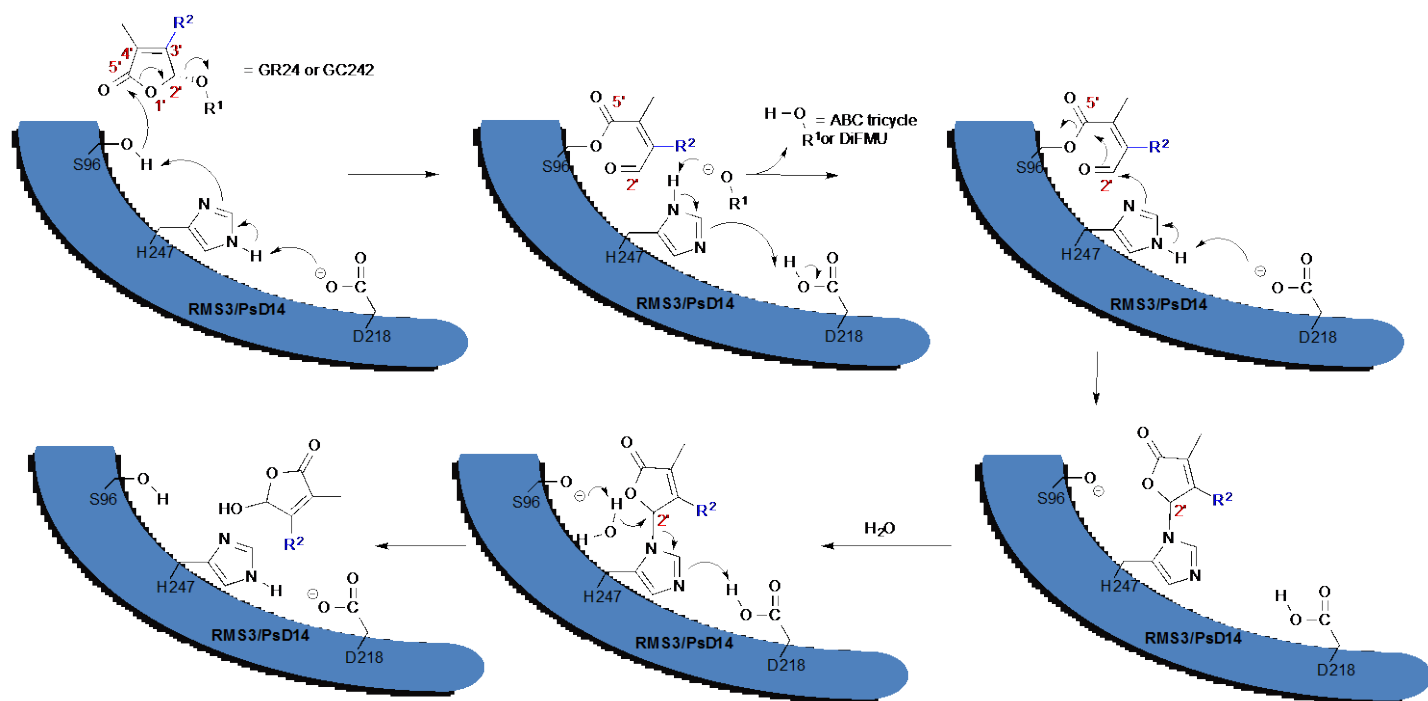
Supplementary Figure 22. Characterization and quantification of the peptide 265-281 after incubation with (±)-GC242. (a) Chromatographic separation of the LysC/trypsin digest of the complex RMS3-complex after incubation with (±)-GC242. **a1**: total ion current of the MS1 track; **a2**: extracted chromatogram of the ion 1,184.6 corresponding to the three charged ion of peptide 265-281 with an adduct of 110 Da; **a3**: extracted chromatogram of the ion 1,147.9 corresponding to the three charged ion of peptide 265-285. AA: calculated area of the mass selected. 91% of the peptide is modified. (b) Zoom on the three charged ion of peptide 265-281 with an adduct of 110 Da. (c) Zoom on the three charged ion of peptide 265-281 corresponding to a deconvoluted mass of 3440.7364 Da. The deconvoluted mass found is 3550.7771 Da. Theoretical exact mass of peptide 265-281 is 3,440.7364 Da, theoretical exact mass of the adduct is 110.0362, theoretical mass of the complex: 3,550.7726. Difference between theoretical mass and experimental mass: 1.4 ppm. Identification of peptide was also confirmed by MS/MS spectrum.



Supplementary Figure 23. Mass spectra of RMS3^{H247A} and RMS3^{S96C} mutant proteins after incubation with (±)-GR22 or (±)-GC242; mass spectra of AtD14 and AtD14-D-ring complex after incubation with (±)-GC242 and characterization and quantification of the peptide 246-264 after incubation with (±)-GC242. (a) Deconvoluted electrospray ionization spectrum of RMS3^{H247A}. (b) Deconvoluted electrospray mass spectrum of RMS3^{H247A} after incubation with (±)-GR24 (500 μM final concentration). (c) Deconvoluted electrospray mass spectrum of RMS3^{H247A} after incubation with (±)-GC242 (500 μM final concentration). (d) Deconvoluted electrospray ionization spectrum of RMS3^{S96C}. (e) Deconvoluted electrospray mass spectrum of RMS3^{S96C} after incubation with (±)-GR24 (500 μM final concentration). (f) Deconvoluted electrospray mass spectrum of RMS3^{S96C} after incubation with (±)-GC242 (500 μM final concentration). (g) Deconvoluted electrospray ionization spectrum of AtD14. (h) Deconvoluted electrospray mass spectrum of AtD14 after incubation with (±)-GR24 (500 μM final concentration). (i) AtD14 amino acid sequence. (j) Mass spectrum of the peptide 246-264 obtained after digestion of AtD14 + (±)-GC242 by LysC/trypsin. [a.u.], arbitrary unit.

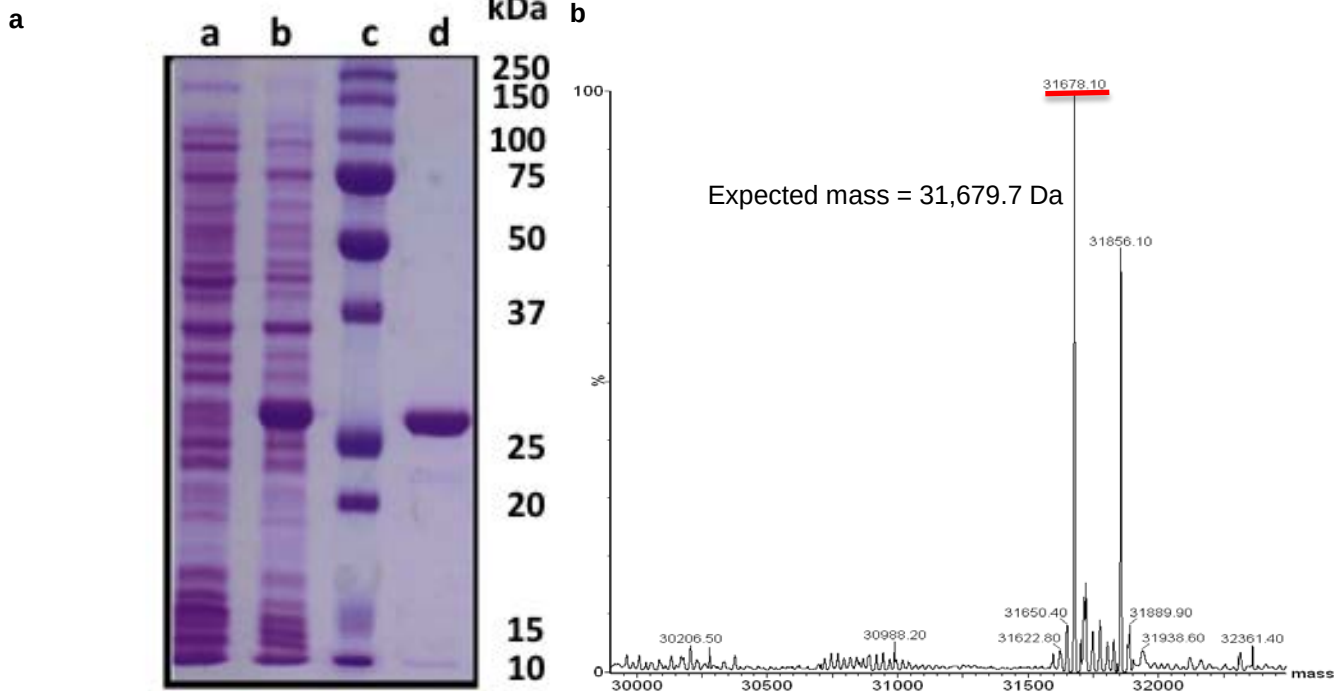


Supplementary Figure 24. Mass spectra of RMS3 and RMS3-D-ring complex after incubation with (±)-GC242 at different pH. (a) Deconvoluted mass spectrum of RMS3 at pH 7.7. (b) Deconvoluted electrospray mass spectrum of RMS3 after incubation with (±)-GC242 (500 μM final concentration) at pH 7.7. The main peaks match with the theoretical mass of RMS3 : $31,679.7 + 110.0 = 31,789.7$ Da and $31,856.0 + 110.0 = 31,964.0$ Da. (c) Deconvoluted mass spectrum of RMS3 after addition of (±)-GC242 (500 μM final concentration) at pH 6.8 and then submitted to incubation 40 min at pH 5.2.

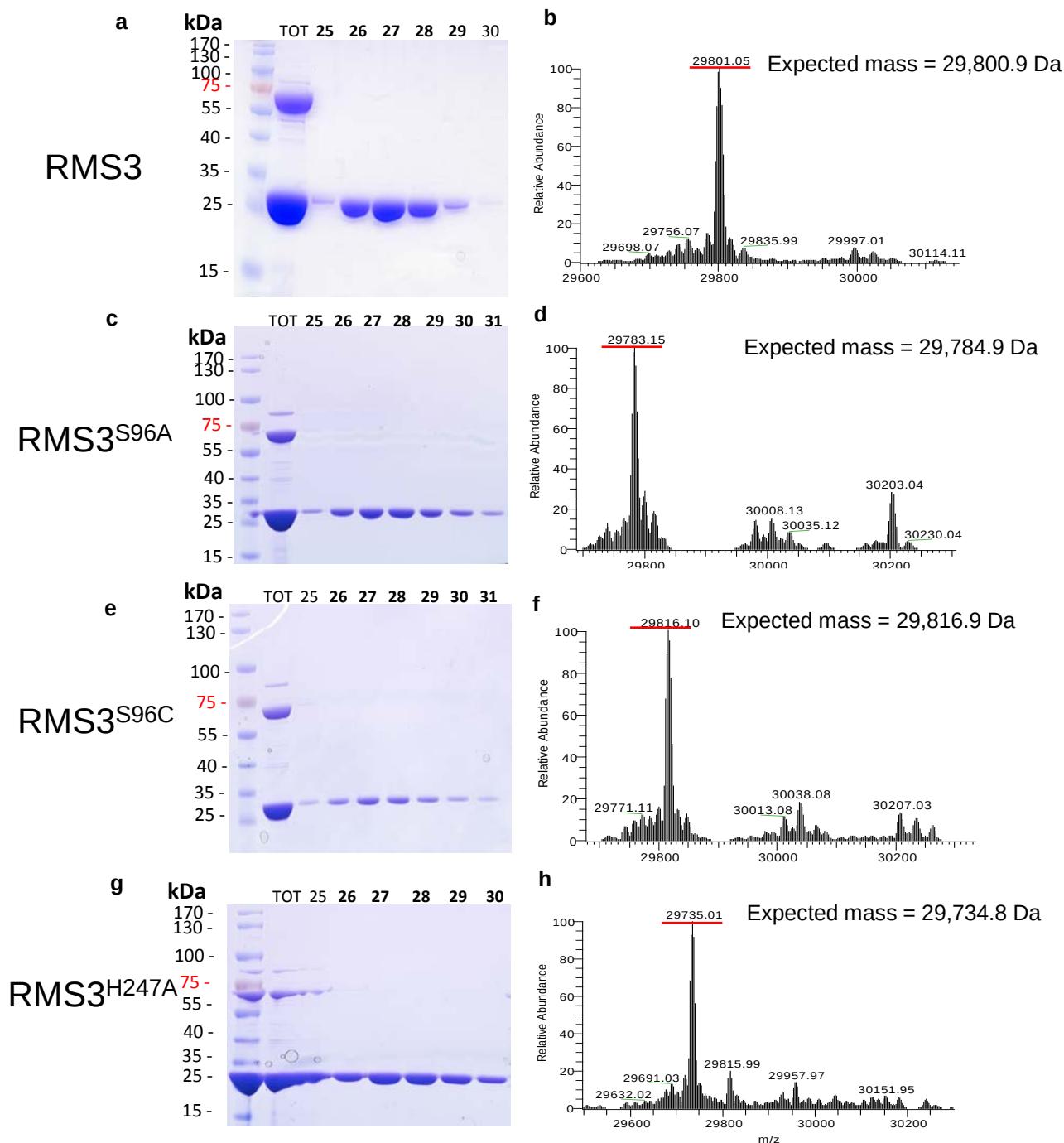


Supplementary Figure 25. Schematic diagram of the proposed mechanism.

RMS3-His



Supplementary Figure 26. RMS3-His protein characterization. (a) SDS-PAGE analysis of expression and purification of recombinant RMS3-His protein. 16% acrylamide gel was used with the Laemmli buffer system (Bio-Rad). Protein bands were visualized by Coomassie brilliant blue staining. a : whole cell extract before induction. b : whole cell extract 23 h post induction by 0.2 mM IPTG at 20 °C. c : Bio-Rad precision Plus Protein All Blue standards (mixture of ten blue-stained recombinant proteins (10–250 kD), including three reference bands (25, 50, and 75 kD). d : recombinant RMS3 protein purified using nickel ion affinity chromatography. (b) Deconvoluted electrospray mass spectrum of RMS3-His. The apparent relative molecular mass of the purified protein (underlined in red) is in accordance with that expected for the protein.

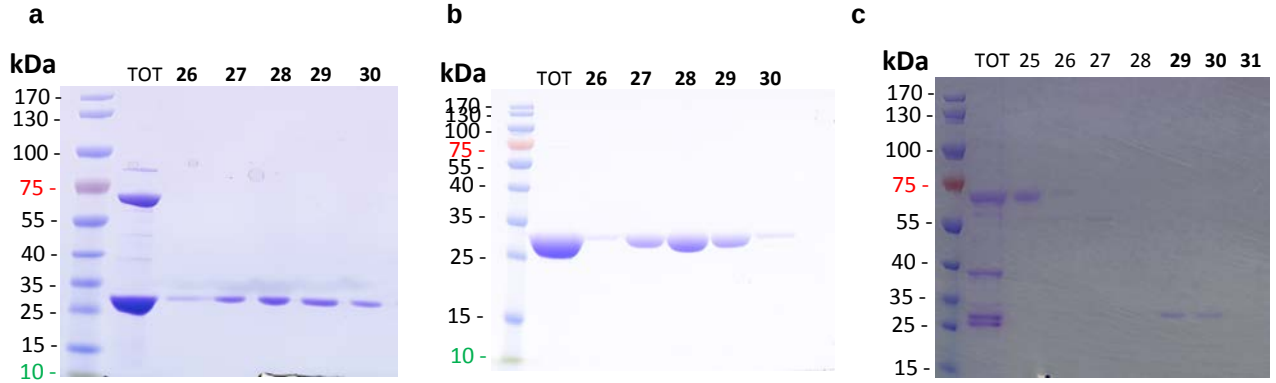


Supplementary Figure 27. Heterologous expression in *Escherichia coli* and gel filtration purification of RMS3 and mutant proteins. Proteins cloned in pGEXT-4T-3, were expressed in *E. coli* as N-terminal GST tagged protein and purified using glutathione Sepharose 4B resin (GE Healthcare). After overnight HRV3C protease digestion, the eluted protein was purified by gel filtration. 15 μ L of protein fractions eluted as a sharp single peak (the number indicate the fraction collected) and total protein extract before gel filtration (TOT) were separated on a 4-12% acrylamide TRIS-glycine gel, and stained with Coomassie Brilliant Blue (**a,c,e,g**). The predicted size of proteins is 28 to 31 kDa. Fractions in bold containing protein, were combined and used for various assays. Approximate molecular weights of protein standards in kDa (PageRuler™ Prestained Protein Ladder, 10 to 180 kDa cat. #26616) are shown on the left. (**b,d,f,h**) Deconvoluted electrospray mass spectra of RMS3, RMS3^{S96A}, RMS3^{S96C} and RMS3^{H247A}. The apparent relative molecular mass of the purified protein (underlined in red) is in accordance with that expected for each protein.

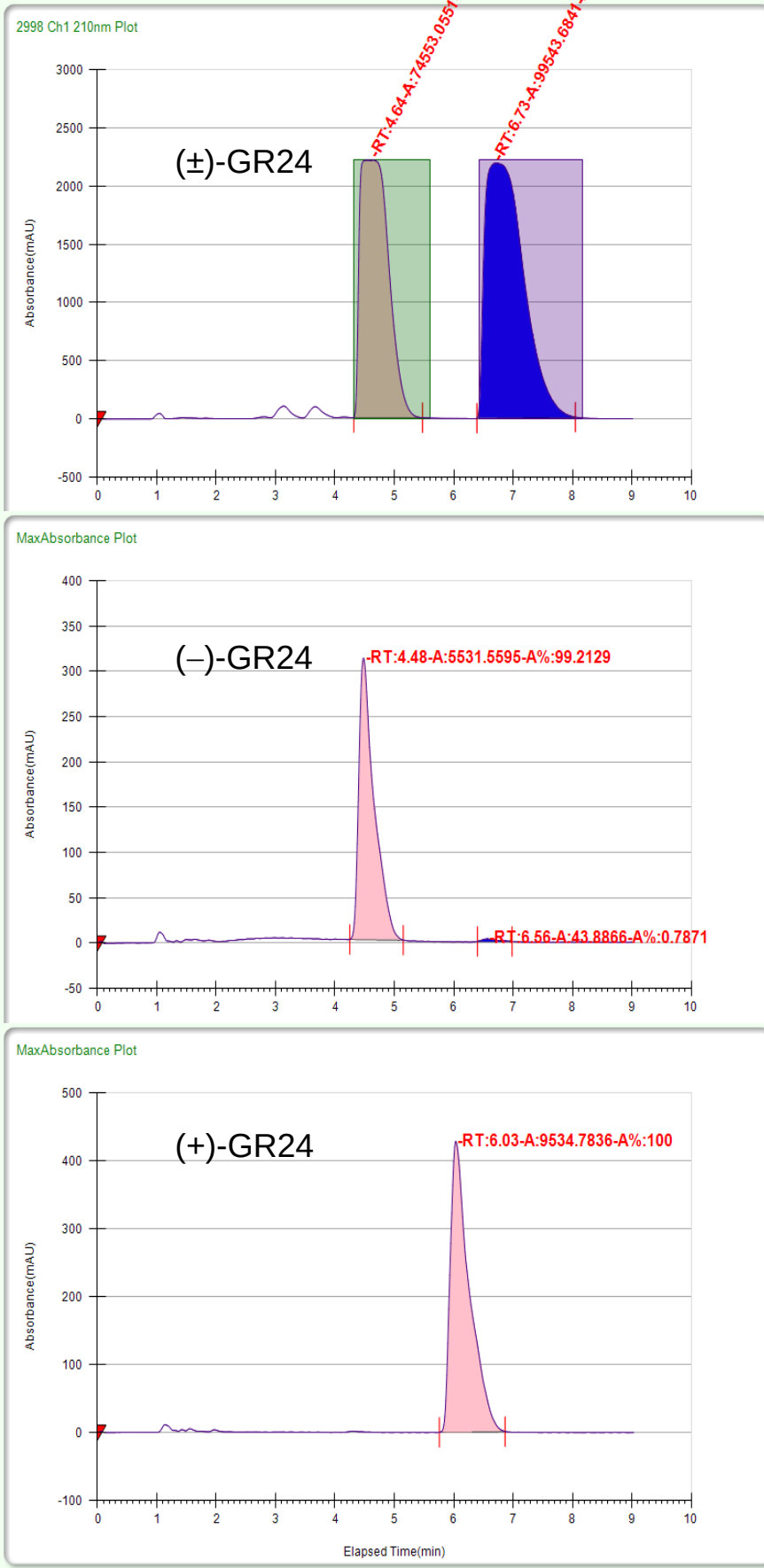
AtD14

HTL

AtMES9



Supplementary Figure 28. Heterologous expression in *Escherichia coli* and gel filtration purification of AtD14, HTL and AtMES9 proteins. Proteins cloned in pGEXT-4T-3, were expressed in *E. coli* as N-terminal GST tagged protein and purified using glutathione Sepharose 4B resin (GE Healthcare). After overnight HRV3C protease digestion, the eluted protein was purified by gel filtration. 15 μ L of protein fractions eluted as a sharp single peak (the number indicate the fraction collected) and total protein extract before gel filtration (TOT) were separated on a 4-12% acrylamide TRIS-glycine gel, and stained with Coomassie Brilliant Blue (**a-c**). The predicted size of proteins is 28 to 31 kDa. Fractions in bold containing protein, were combined and used for various assays. Approximate molecular weights of protein standards in kDa (PageRuler™ Prestained Protein Ladder, 10 to 180 kDa cat. #26616) are shown on the left.



Supplementary Figure 29. Chiral HPLC of (±)-GR24 and of (-)-GR24 and (+)-GR24 after separation.

References:

- ¹ Schwarz, M. & Dayhoff, M. (ed Dayhoff M) 353-358 (National Biomedical Research Foundation, Washington, DC, 1979).
- ² Tamura, K. *et al.* MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* **28**, 2731-2739, (2011).
- ³ Kagiya, M. *et al.* Structures of D14 and D14L in the strigolactone and karrikin signaling pathways. *Genes Cells* **18**, 147-160, (2013).