Glycosyltransferase UGT76E1 forms 12-*O*-glucosylpyranosyl-jasmonic acid in wounded *Arabidopsis thaliana* leaves

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Running title: *UGT76E1 glycosylates 12-hydroxy-jasmonic acid*

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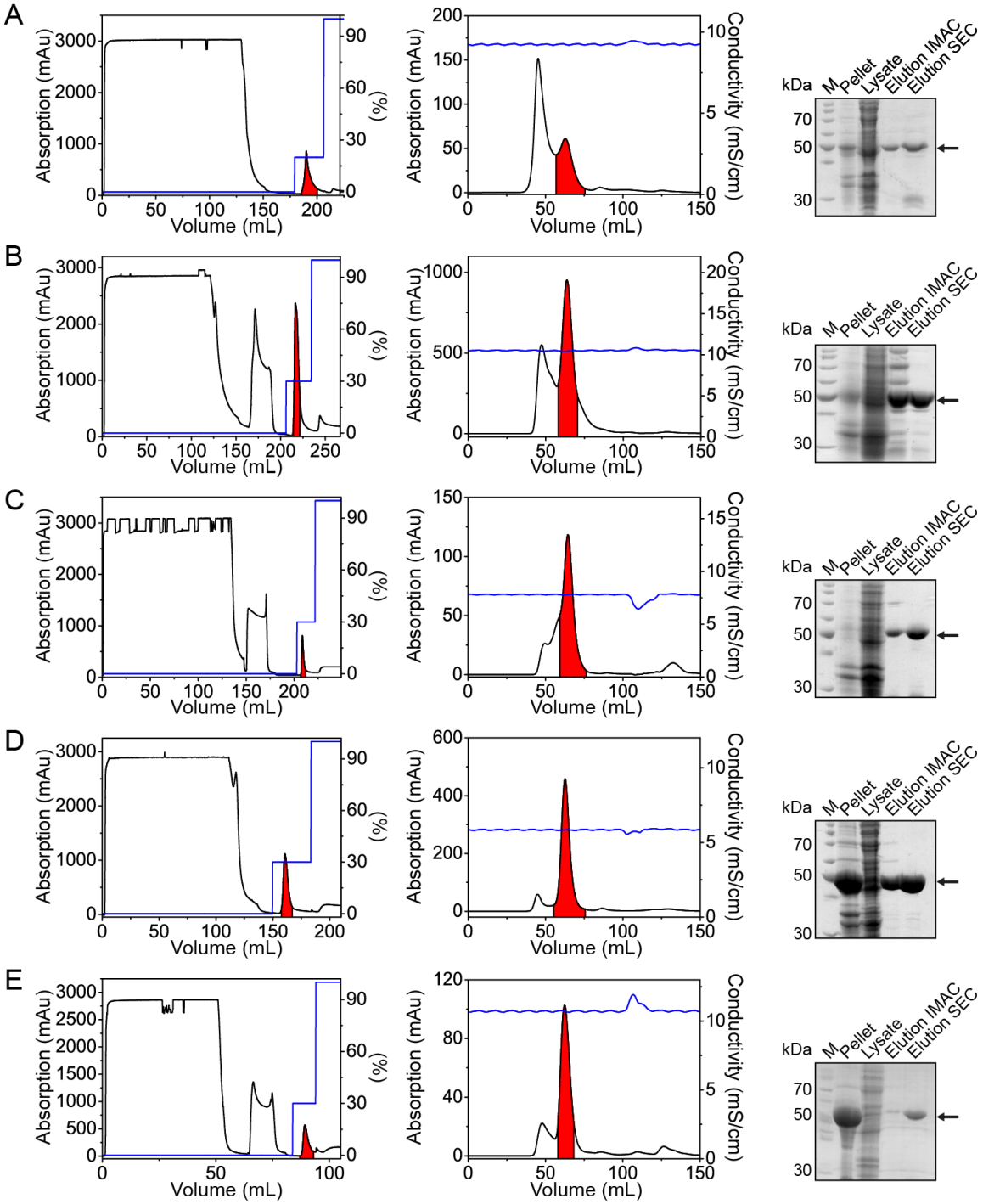

Figure S1: Protein purification of UGT76E1, UGT76E2, UGT7E11, UGT76E12, and UGT74F1
UGTs fused to an N-terminal His-tag and heterologously expressed in *E. coli* BL21 Star (DE3) were purified by a two-step protein purification strategy of immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC). Shown are the chromatograms for the respective IMAC- and SEC-purification steps and a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with samples of pellet, lysate, IMAC eluate, and SEC eluate. Arrows indicate the size of the respective purified protein. Chromatograms illustrate the absorption at 280 nm (milli absorption units (mAU)) during elution. For IMAC, the second y-axis shows the concentration of elution buffer (%). For SEC, the second y-axis shows the conductivity (mS/cm). The fraction that contained the protein of interest is marked in red. Purification of **A** UGT76E1 **B** UGT76E2 **C** UGT76E11 **D** UGT76E12 **E** UGT74F1. The depicted purification is representative for at least four independent purifications for each UGT.

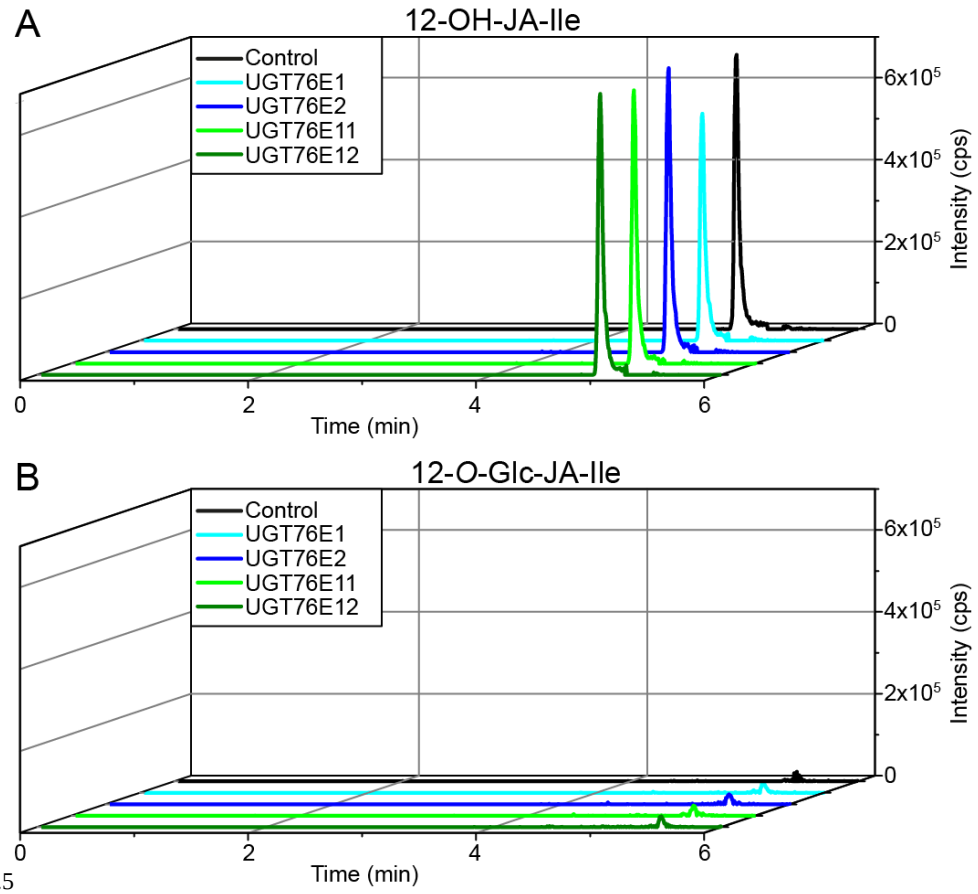

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Figure S2: 12-hydroxy-jasmonoyl-isoleucine can be detected in the quasi-native substrate mixture, but is not accepted as substrate for glycosylation by UGT76E1, UGT76E2, UGT7E11, UGT76E12, or UGT74F1

Plants were grown for six weeks under short day conditions. Leaves were wounded three times across the mid vein by squeezing with forceps. Damaged rosette leaves were harvested at 5 hpw (pool of 10 plants per sample) and directly frozen in liquid nitrogen for subsequent extraction of metabolites. Metabolite extracts were resolved in 50 mM Tris pH 8, 100 mM NaCl buffer and the assay was performed with 0.1 mM UDP-Glc and 100 µg of the indicated active UGT-enzymes or inactive enzyme (control) for 1 h at 25 °C. The reactions were stopped by adding acetonitrile and analyzed by LC-MS. Given are the extracted ion chromatograms of **A** 12-hydroxy-jasmonoyl-isoleucine (12-OH-JA-Ile) in the negative ionization mode ($[M-H]^-$ 338.1967) and of **B** 12-*O*-glucosyl-jasmonoyl-isoleucine (12-*O*-Glc-JA-Ile as a possible UGT-product) in the negative ionization ($[M-H]^-$ 500.2496). 12-*O*-Glc-JA-Ile is not detectable in *A. thaliana* leaves 5 hpw. All chromatograms show relative signal intensities fixed to 7 x 10^7 counts per second (cps) over time. The data are representative for three measurements of one experiment.

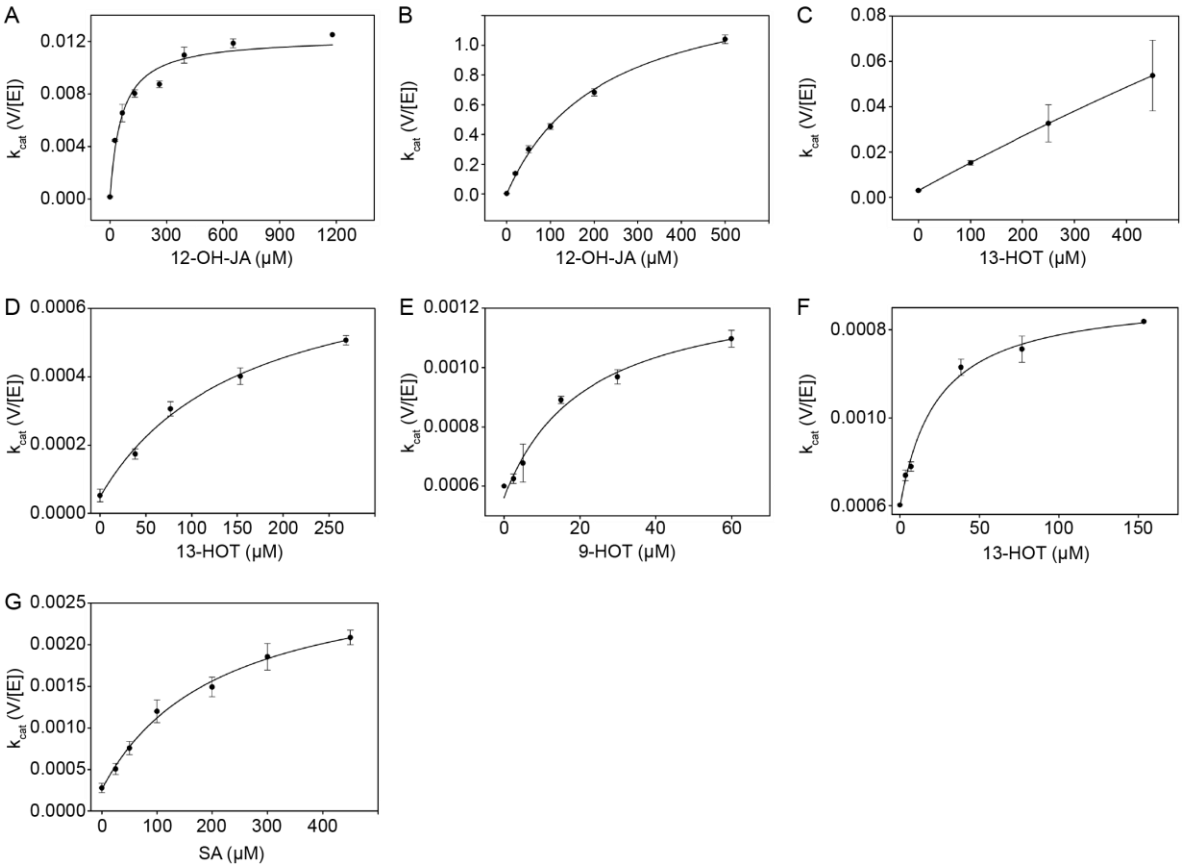


Figure S3: Enzyme kinetics with the preferred substrates

The enzymatic parameters for UGT76E2, UGT76E11, UGT76E12, and UGT74F1 were determined by spectrophotometric assay. The UGT-reactions were coupled via the co-product UDP to the pyruvate kinase and the lactate dehydrogenase in 1 : 1 : 1 stoichiometry. The reactions were performed with 5 µg of the respective UGT in gel filtration buffer, 0.5 mM UDP-Glc, and indicated substrate concentrations at 25 °C. The reaction was monitored at 340 nm for 600 s. Kinetics and Michaelis-Menten fit for **A** UGT76E1 with 12-hydroxy-jasmonic acid (12-OH-JA, (due to limitations in 12-OH-JA availability, the value for 1200 µM was measured once only) **B** UGT76E2 with 12-OH-JA (due to limitations in 12-OH-JA availability, the value for 500 µM was measured twice) **C** UGT76E11 with 13-hydroxy-octadecatrienoic acid (13-HOT). **D** UGT76E12 with 9-hydroxy-octadecatrienoic acid (9-HOT). **E** UGT76E12 with 13-HOT (due to limitations in 13-HOT availability, the value for 150 µM was measured only once). **E** UGT74F1 with salicylic acid (SA). If not stated otherwise, the data are mean values with standard deviation of three biological replicates.

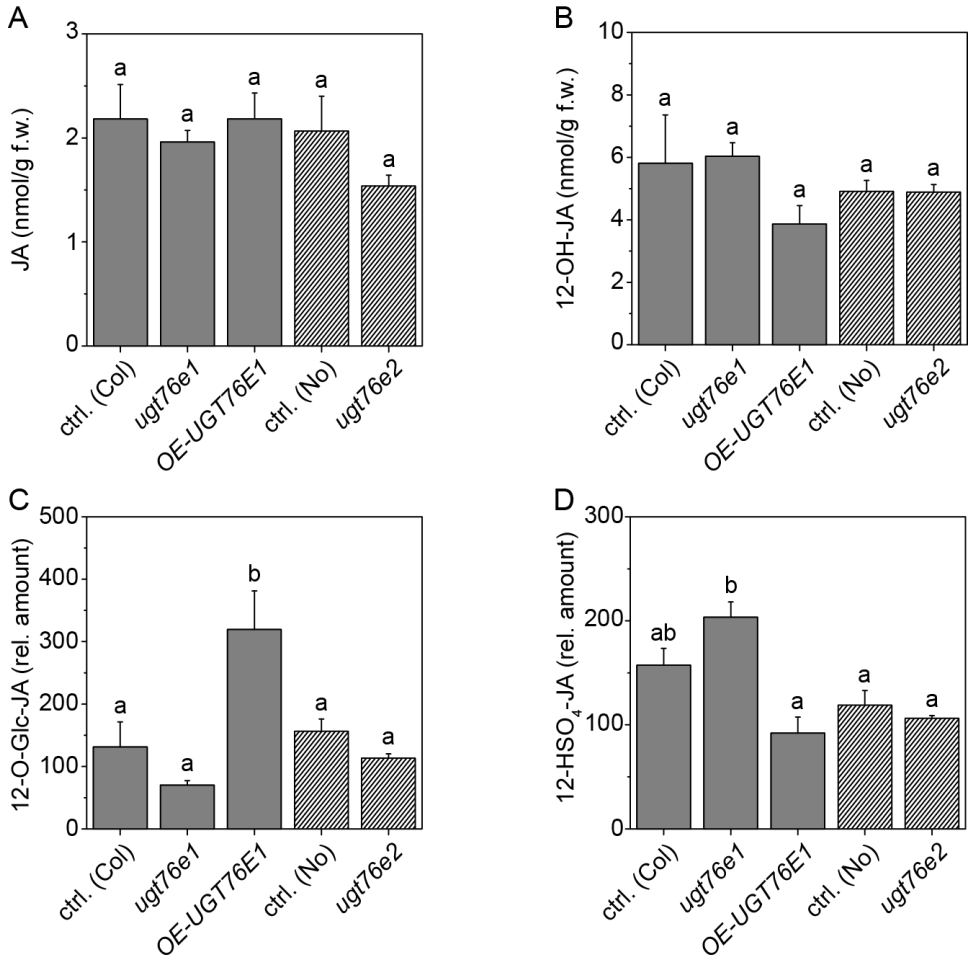


Figure S4: Jasmonate profiles of *A. thaliana* after wounding
Metabolite levels of the Columbia 0 WT (ctrl. (Col)), a respective *UGT76E1*-CRISPR/Cas-mutant (*ugt76e1*), an overexpresser of *UGT76E1* (*OE-UGT76E1*) (grey bars) as well as the Nossen control (ctrl. (No)) and the respective *ugt76e2* mutant (striped bars) were determined. For the wounding experiment, leaves were wounded three times across the mid vein. Damaged rosette leaves were harvested at 5 hours post wounding, extracted, and analyzed by LC-MS/MS. Quantitative data are given in nmol/g fresh weight for **A** jasmonic acid (JA), **B** jasmonoyl-isoleucine (JA-Ile), and **D** 11/12-hydroxy-JA (11/12-OH-JA). Relative amounts were given for **C** 12-hydroxy-JA-Ile (12-OH-JA-Ile), **E** 12-*O*-glucosyl-JA (12-*O*-Glc-JA), and **F** 12-hydroxy-JA sulfate (12-SO_4-JA). The data show means of 3-6 biological replicates + SEM and one-sided ANOVA-test (p=0.05, turkey test).

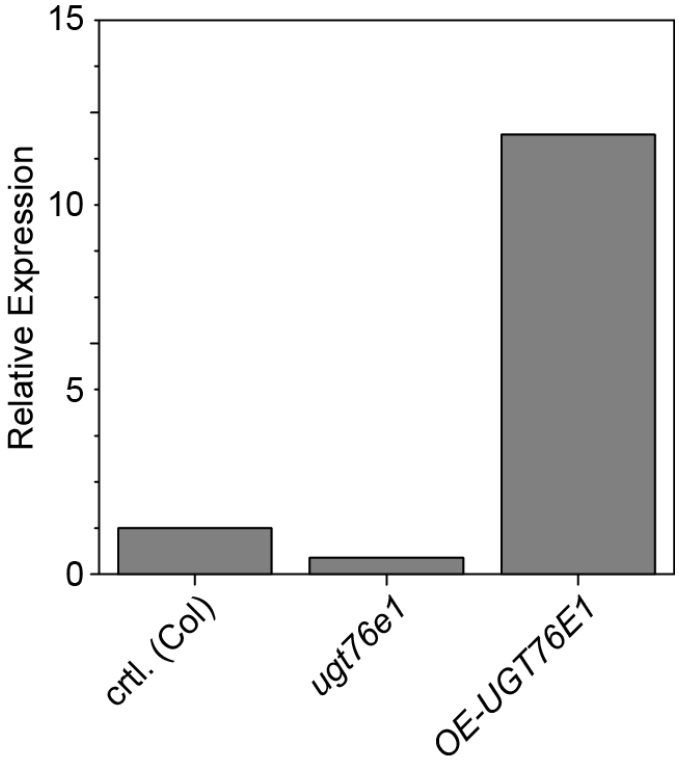


Figure S5: Mutation of *UGT76E1* effects its expression
Quantitative real-time PCR of *UGT76E1* in Columbia 0 WT (ctrl. (Col)), a respective *UGT76E1*-CRISPR/Cas-mutant (*ugt76e1*), and an overexpresser of *UGT76E1* (*OE-UGT76E1*) 2 hours post wounding. Plants were grown for six weeks under short day conditions and wounded. RNA was isolated. All expression values are normalized to *Actin 8* as reference and to the WT. Each data point represents the mean of 1 to 3 biological replicates.

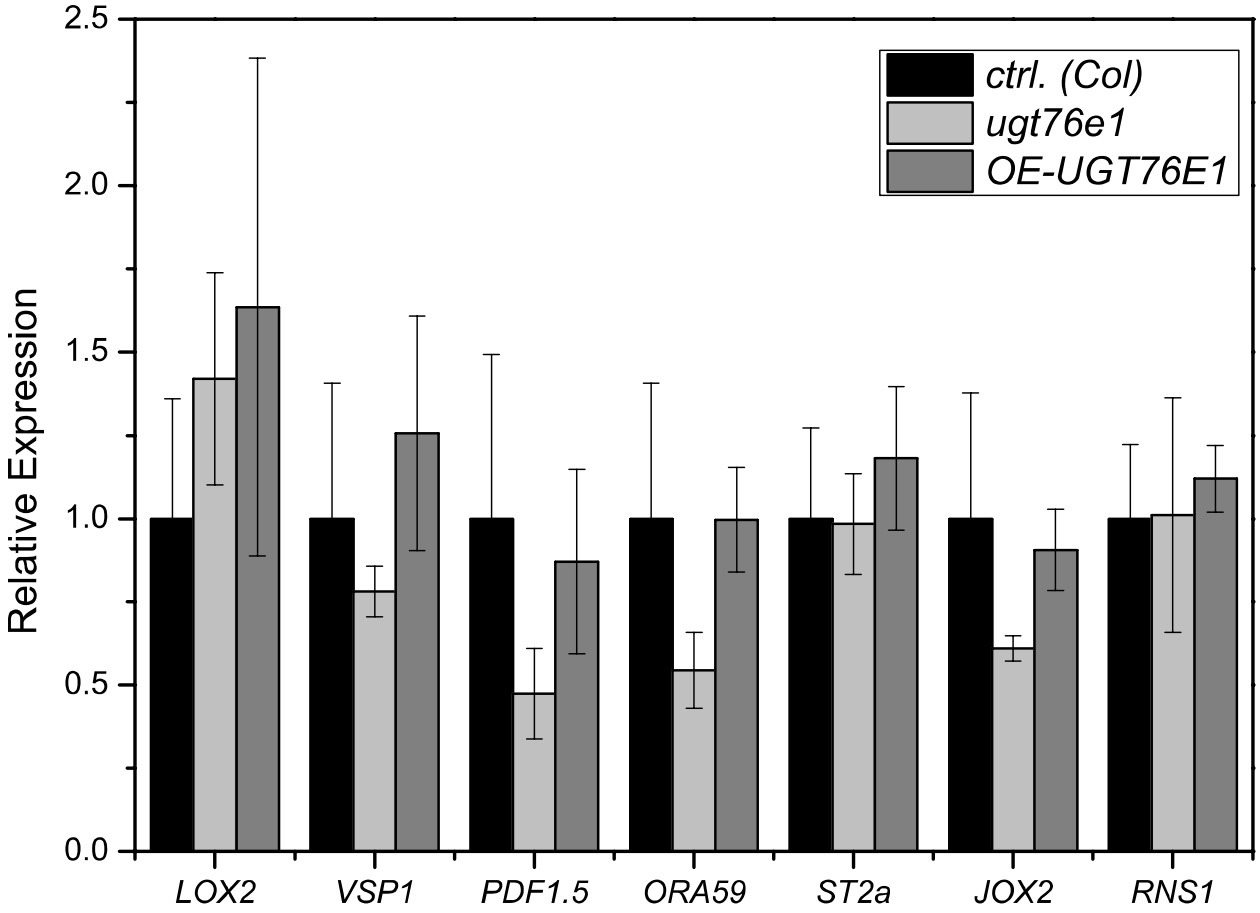


Figure S6: Gene expression of jasmonate signaling-related genes is not changed in wounded leaves of mutant plants with altered *UGT76E1* expression

Quantitative real-time PCR of *LOX2 (LIPOXYGENASE2), VSP1 (VEGETATIVE STORAGE PROTEIN1), PDF1.5 (PLANT DEFENSIN 1.5), ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF gene 59), ST2a (SULFOTRANSFERASE a), JOX2 (JASMONATE OXIDASE2)* and *RNS1 (RIBONUCLEASEI)* in wounded leaves in Columbia 0 WT (ctrl. (Col)), an *UGT76E1*-CRISPR/Cas-mutant (*ugt76e1*), and an overexpressor of *UGT76E1* (*OE-UGT76E1*). Plants were grown for six weeks under short day conditions. Rosette leaves were wounded three times across the mid vein and harvested straight into liquid nitrogen after 5 hours. RNA was isolated. All expression values are normalized to *Actin 8* as reference and to the WT. Each data point represents the mean of 3 to 6 biological replicates with standard error.

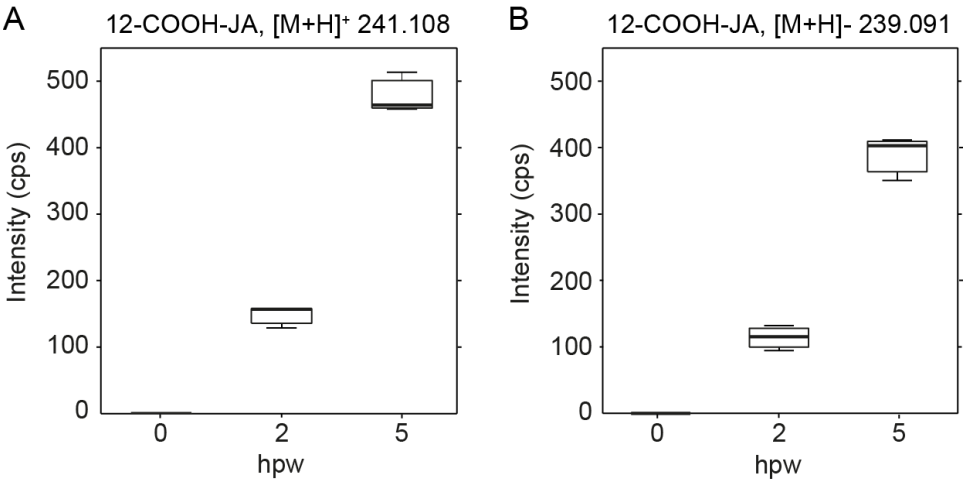


Figure S7: 12-carboxy-JA was identified in leaves after wounding

The metabolite 12-carboxy-JA (12-COOH-JA) was identified in the *ex vivo* metabolite fingerprinting analysis. Plants were wounded three times across the mid vein. Damaged rosette leaves were harvested at 0, 2, and 5 hours post wounding (hpw), extracted, and used as substrate mix for the *ex vivo* activity assay. The extracts were resolved in 50 mM Tris pH 8, 100 mM NaCl buffer and 20 µl acetonitrile. Samples were analyzed by mass spectrometry with a method developed for non-targeted fingerprinting. The particular features of 12-COOH-JA were found in both the **A** positive ionization mode and **B** in the negative ionization mode shown as Box-Whisker-plots. The data represent three measurements of one experiments. 10 plants were pooled for each time point of one experiment. Data were analyzed with the MarVis tool.

Table S1: Calculated protein parameters and predicted localizations for UGT76E1, UGT76E2, UGT76E11, UGT76E12 and UGT74F1

The table depicts molecular weight (MW), specific extinction coefficient at 280 nm (ε), theoretical isoelectric point (pI), presents of a signal peptide (SignalP), target peptides (TargetP), and the number of transmembrane domains (TMHMM) for UGT76E1, UGT76E2, UGT76E11, UGT76E12, and UGT74F1. The protein parameters were calculated with ProtParam and localization predictions were done with SignalP, TargetP (plant settings), and TMHMM online tools. Access was 04.05.2018. Reaction optima were determined in reactions of 0.1 mM ω-hydroxy-hexadecanoic acid and 0.5 mM UDP-Glc incubated with 30 µg UGT at 25 °C for 1 h. Optimal reaction temperature (opt. Temp.) was determined in the range of 4 to 50 °C and optimal pH-values (opt. pH) were determined between pH 3 – 11 in the Britton-Robinson buffer system. The best product formation was detected by LC-MS and given here. Data represent at least three independent experiments for every enzyme.

Parameter	**UGT76E1**	**UGT76E2**	**UGT76E11**	**UGT76E12**	**UGT74F1**
MW (kDa)	50.8	50.1	50.6	51.7	50.3
ε ($kM^{-1}cm^{-1}$)	66.4	63.5	58.0	55.5	62.4
pI	6.6	5.4	5.9	6.0	5.5
SignalP	/	/	/	/	/
TargetP	mitochondrial	/	/	/	/
TMHMM (no.)	0	0	0	0	0
Opt. Temp (° C)	20	30	25	30	/
Opt. pH	7.5	7.5	8.0	8.0	/

Table S2: Chemical structures of the substrates of the radiolabeled specificity assay
Chemical structures of hexadecanoic acid (16:0), ω-hydroxy-16:0 (ω-OH-16:0), 2-hydroxy-16:0 (2-OH-16:0), 3-hydroxy-16:0 (3-OH-16:0), hexadecanol (OH-C16), benzoic acid (BA), salicylic acid (SA), pipecolic acid (Pip), indole-3-caboxylic acid (ICA), abscisic acid (ABA), zeatin, gibberellic acid (GA), 12- 12-oxo-phytodienoic acid (12-OPDA), JA, 12-hydroxy-JA (12-OH-JA), 11-hydroxy-JA (11-OH-JA), 12-hydroxy-JA-methyl ester (12-OH-JA-ME), quercetin, dihydro-myricetin (dh-myricetin), and dihydro-kaempferol (dh-kaempferol).

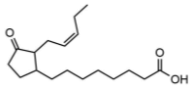
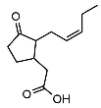
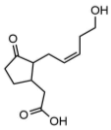
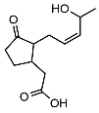
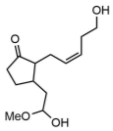
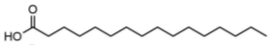
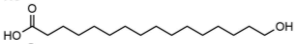
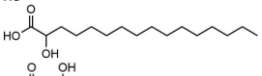
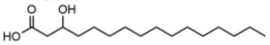
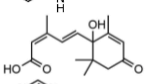
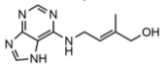
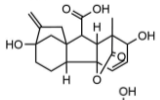
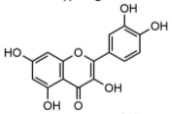
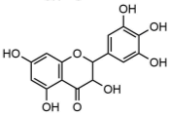
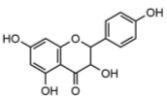
	Substrate	Structure
1	12-OPDA	
2	JA	
3	12-OH-JA	
4	11-OH-JA	
5	12-OH-JA-ME	
6	16:0	
7	ω-OH-16:0	
8	2-OH-16:0	
9	3-OH-16:0	
10	OH-C16	
11	BA	
12	SA	
13	Pip	
14	ICA	
15	ABA	
16	Zeatin	
17	GA	
18	Quercetin	
19	Dh-Myricetin	
20	Dh-Kaempferol	

Table S3: Confirmation of the chemical structure of UGT-substrates and products by high-resolution LC-MS/MS analyses

ID	Marker	RT (min)	Exact mass (Da)	detected ion	Sum furmular	Ref.	identity conf. by	Specific fragments	CE (eV)
1	12-OH-JA	3.52	226.1205	M-H	C12H18O4		B, D	$[M-H]^-$ 225.1132, 59.0128	12
2	12-*O*-Glc-JA	3.24	388.1733	M-H	C12H18O4		B, D	$[M-H]^-$ 387.1608, 207.0981 $[M-Glc-H_2O]^-$, 59.0122	25
3	SA	4.17	138.0316	M-H	C12H18O4	MID 616	A	$[M-H]^-$ 137.0221, 93.0330	12
4	2-*O*-Glc-SA	2.72	300.0845	M-H	C12H18O4	MID 616	C	$[M-H]^-$ 299.0756, 137.0225 $[M-Glc]^-$, 93.0328	12
5	ω-OH-16:0	6.72	272.2351	M-H	C12H18O4	MID 85386	A, D	$[M-H]^-$ 271.2267, 253.218, 225.2191, 59. 0139	32
6	ω-*O*-Glc-16:0	5.81	434.2879	M-H	C12H18O4	MID 85386	C	$[M-H]^-$ 433.2742, 415.2679, 271.2281 $[M-Glc]^-$, 59.0139	25
7	UDP-glucose	0.72	566.0550	M-H	C12H18O4	MID 97	A	$[M-H]^-$ 565.0449, 384.9825, 323.0267, 241.0108, 78.9583	18
8	UDP	0.86	404.0021	M-H	C12H18O4	MID 5886	A	$[M-H]^-$ 402.9882, 305.0114, 272.9530, 158.9226, 111.017	20
9	11-HHT	6.10	266.1881	M-H	C12H18O4		A	$[M-H]^-$ 265.1790, 247.1695, 195.1026, 167.1083	18
10	11-*O*-Glc-HHT	5.63	428.2410	M-H	C12H18O4		C	$[M-H]^-$ 427.23129, 265.1785 $[M-Glc]^-$, 247.1681	25
11	13-HOT	7.11	294.2194	M-H	C12H18O4		A, D	$[M-H]^-$ 293.2117, 275.162	15
12	13-*O*-Glc-HOT	6.05	456.2723	M-H	C12H18O4		C	$[M-H]^-$ 455.2660, 293.2117 $[M-Glc]^-$, 275.1950, 223.1275	25
13	C11H18O3	6.01	198.1255	M+H	C12H18O4			$[M+H]^+$ 199.1888, 181.1221, 125.0595, 111.0438, 55.0541	12
14	C11H18O3-Glc	5.01	360.1784	M+NH4	C12H18O4			$[M+H]^+$ 378.2452, 199.1327$[M-Glc]^-$, 181.1221, 163.1112, 111.0440	18
15	12-COOH-JA	4.31	240.0998	M-H M+H	C12H18O4		A	$[M-H]^-$ 239.095, 195.105, 59.013	12
16	12-OH-JA-Ile	4.94	339.2045	M-H M+H	C12H18O4		E		

A	MS/MS fragment information from literature/data base
B	MS/MS fragment information from identical standard
C	MS/MS fragment information of non-glycosylated standard
D	Co-elution of authentical standard
E	Exact mass measurement only

Table S4: Buffer concentrations for protein purifications

Enzyme	Buffer		
UGT76E1	HisTALON		
	50	mM	Tris/HCl pH 9.0
	100	mM	NaCl
	0.1	%	Tween20
	200	mM	Imidazol (buffer B)
	Gelfiltration		
	50	mM	Tris/HCl pH 9.0
	100	mM	NaCl
UGT76E2	HisTrap		
	50	mM	Tris/HCl pH 8.0
	100	mM	NaCl
	0.1	%	Tween20
	500	mM	Imidazol (buffer B)
	Gelfiltration		
	50	mM	Tris/HCl pH 8.0
	100	mM	NaCl
UGT76E11	HisTrap		
	20	mM	Tris/HCl pH 7.5
	50	mM	NaCl
	0.1	%	Tween20
	10	%	Glycerol
	500	mM	Imidazol (buffer B)
	Gelfiltration		
	20	mM	Tris/HCl pH 7.5
	50	mM	NaCl
UGT76E12	HisTrap		
	20	mM	Tris/HCl pH 7.5
	50	mM	NaCl
	10	%	Glycerol
	500	mM	Imidazol (buffer B)
	Gelfiltration		
	20	mM	Tris/HCl pH 7.5
	50	mM	NaCl
UGT74F1	HisTrap		
	50	mM	Tris/HCl pH 7.5
	100	mM	NaCl
	0.1	%	Tween20
	500	mM	Imidazol (buffer B)
	Gelfiltration		
	50	mM	Tris/HCl pH 7.5
	100	mM	NaCl
Wash buffer	HisTrap		
	50	mM	Tris/HCl pH 7.5
	100	mM	NaCl
	2	mM	DTT
	5	mM	ATP
	5	mM	MgCl2
	20	mM	Imidazol

Table S5: Oligonucleotides used in this work

Name	Sequence	Item	rest. site
UGT76E1-fwd	ACGCATATGATGGAAGAACTAGGAGTGAAGAGAAG	Cloning / expression	NdeI
UGT76E1-rev	ACGCTCGAGGTGAACAATGATTTTGTCTATAAATGC	Cloning / expression	XhoI
UGT76E2-fwd	ACGCATATGGAGGAAAAGCAAG	Cloning / expression	NdeI
UGT76E2-rev	ACGCTCGAGCATGGAATTAAC	Cloning	XhoI
UGT76E11-fwd	ACGCATATGGAGGAAAAGCC	Cloning	NdeI
UGT76E11-rev	ACGCTCGAGTAGAGTCCTCATG	Cloning	XhoI
UGTE12-fwd	ACGCATATGATGCAGGTTTTGGGAATGGAGG	Cloning	NdeI
UGTE12-rev	ACGCTCGAGTCATAGAGTCCTTATGAAGTGTAC	Cloning	XhoI
UGT74F1-fwd	ACGGAATTCGAGAAGATGAGAGG	Cloning	EcoRI
UGT74F1-rev	ACGCTCGAGTCATTTGATTTGAATTT	Cloning	XhoI
E1-q-left	TGCCAACTTCAGCATTTGGG	qPCR	
E1-q-right	ACCATGCCAAAGATGAGCTC	qPCR	
E2-q-left_new	GAGCTCATCTTTGGCAAGGTTG	qPCR	
E2-q-right_new	TTCAACCACTCAACGCAACTC	qPCR	
E11-q-left	TTTTGGAGCCATTGCGGATG	qPCR	
E11-q-right	ATCACTGGAAAACGGCTTGC	qPCR	
E12-q-left	TCGTCACCATTCCAGAAAGC	qPCR	
E12-q-right	TTGTTGCAGCACCAACTGAC	qPCR	
SGT-q-left	AAACCTTCGGCTCCAAAACC	qPCR	
SGT-q-right	ATCCATTGCAAGGTCAAGCG	qPCR	
LOX2Fwd	GGGCGTACTGGTCGTGGTTA	qPCR	
LOX2Rev	TGCACGAGCGTTGATTTCCATG	qPCR	
VSP1F	AGAGCTGGAGCTGGTGTTGTTAA	qPCR	
VSP2F	GAACACCCATTCCGGTAACACCA	qPCR	
PDF1.2Fa	AGAAGTTGTGCGAGAAGCCA	qPCR	
PDF1.2R a	CACTTGTGAGCTGGGAAGACA	qPCR	
ORA59.2-F	GATCAGGCGGCTTTCGCTT	qPCR	
ORA59.2-R	CAGCACCTAAATCCTCAAGAACC	qPCR	
ST2a-for	CTGAGGGCCTACTATATACG	qPCR	
ST2a-rev	CGACAAACTTCGGTGTTGAC	qPCR	
JOX2	CTCATCCCCATGCTTTCATC	qPCR	
JOX2	TCCGAGTTCACTATCACTCTATGC	qPCR	
RNS1_fwd	GTGTTGTTATCCAAATTCAGGC	qPCR	
RNS1_rev	CATGCTTCTCCCATTCGTG	qPCR	
Actin8-RT_for	GGTTTTCCCCAGTGTTGTTG	qPCR	
Actin8-RT_rev	CTCCATGTCATCCCAGTTGC	qPCR	
U6-29p-F	TTAATCCAAACTACTGCAGCCTGAC	CRISPR-Sequencing	
U6-29-p-R	AGCCCTCTTCTTTCGATCCATCAAC	CRISPR-Sequencing	
U6-1t-F	GCTAAGACAAAGTGATTGGTCCGTT	CRISPR-Sequencing	
U6-1t-R	AACGGACCAATCACTTTGTCTTAGC	CRISPR-Sequencing	
A-DT1-BsF	ATATATGGTCTCGATTGCGGGAAGGCTCTTTACTCCAGTT	CRISPR/Cas9	
A-DT1-F0	TGCGGGAAGGCTCTTTACTCCAGTTTTAGAGCTAGAAATAGC	CRISPR/Cas9	
A-DT0-BsR2	ATATTATTGGTCTCAATCTCTTAGTCGACTCTACCAAT	CRISPR/Cas9	

A-DT2-BsF2	ATATTATTGGTCTCAAGATTGCAGACTCAGTTAAGCTGCCTGTT	CRISPR/Cas9
A-DT2-F0	TGCAGACTCAGTTAAGCTGCCTGTTTTAGAGCTAGAAATAGC	CRISPR/Cas9
A-DT0-BsR3	ATATTATTGGTCTCATCACTACTTCGTCTCTAACCAT	CRISPR/Cas9
A-DT3-BsF3	ATATTATTGGTCTCAGTGATTGCTCTGGTAAGCTTTCTGGAAGTT	CRISPR/Cas9
A-DT3-F0	TGCTCTGGTAAGCTTTCTGGAAGTTTTAGAGCTAGAAATAGC	CRISPR/Cas9
A-DT4-R0	AACAACAGTCCTTGAAGCTCACCAATCACTACTTCGACTCTAGCTGTAT	CRISPR/Cas9
A-DT4-BsR	ATTATTGGTCTCTAAACAACAGTCCTTGAAGCTCAC	CRISPR/Cas9
UGT76E1-detect (rev)	CGGTCAAGCTGCCTGGG	Seq-CRISPR
UGT76E2-detect (rev)	CAGGATCTTTCATGTCG	Seq-CRISPR
UGT76E11-detect (rev)	CGTTTTGTTGTCCTTTGGG	Seq-CRISPR
UGT76E12-detect (rev)	CTTTGGCTGCAGCTTCAGC	Seq-CRISPR
UGT76E2-int-rev	TGACCCCGGAATAGAGCCCGGTCTGAC	Expression

Table S6: CRISPR target sequences for *UGT76E1, UGT76E2, UGT76E11*, and *UGT76E12*
Targets were chosen with the help of three online tools: CRISPRdirect (https://crispr.dbcls.jp/), CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2/), and The Genetic Pertubation Platform for designing sgRNAs for CRISPRko (http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). The best targets are listed which were used integrated into the CRISPR/Cas9 vector.

Target	Sequence	Strand	Off targets	Score	On target efficiency	GC content [%]
UGT76E1	CGGGAAGGCTCTTTACTCCAAGG	+	0	99	0.57	55
UGT76E2	CCCAGGCAGCTTAACTGAGTCTG	−	0	95	0.51	50
UGT76E11	CCATTCCAGAAAGCTTACCAGAG	−	1 (*UGT76E12*)	49	0.51	45
UGT76E12	GGTGAGCTTCAAGGACTGTTTGG	+	0	71	0.39	50