

Figure S1: Overview of the aliphatic and indole glucosinolate biosynthetic pathways. The pathways consist of enzymes specific for either class of glucosinolates as well as common enzymes. Only aliphatic glucosinolate biosynthesis requires chain-elongation steps catalyzed inside plastids.

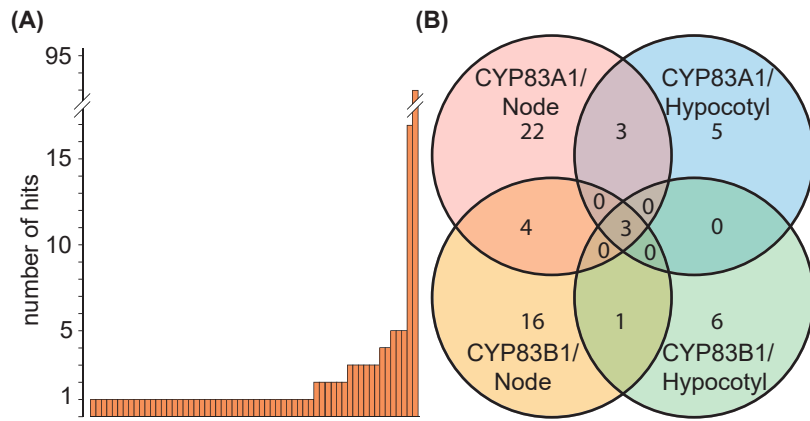


Figure S2: Frequency of identification of 60 candidate interactors identified by yeast 2-hybrid screens **(A)** and Venn diagram illustrating the overlap between results of the four individual screens with CYP83A1 and CYP83B1 baits and prey libraries generated from node or hypocotyl tissues **(B)**.

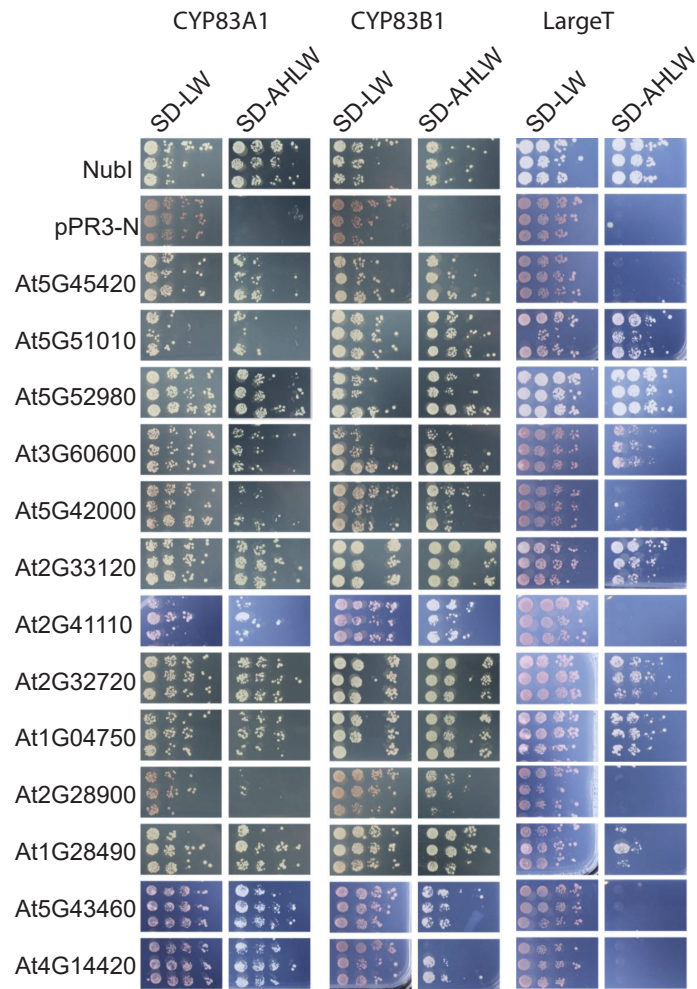


Figure S3: Interaction assay of interactors detected in yeast 2-hybrid screens with bait proteins CYP83A1, CYP83B1 and the unrelated control bait protein LargeT by pairwise co-expression in yeast. Cell suspensions were spotted on SD-LW (growth control) or SD-AHLW (selective medium for interacting protein pairs) medium in dilution series. Nubl: positive control; pPR3-N: empty prey vector, negative control.

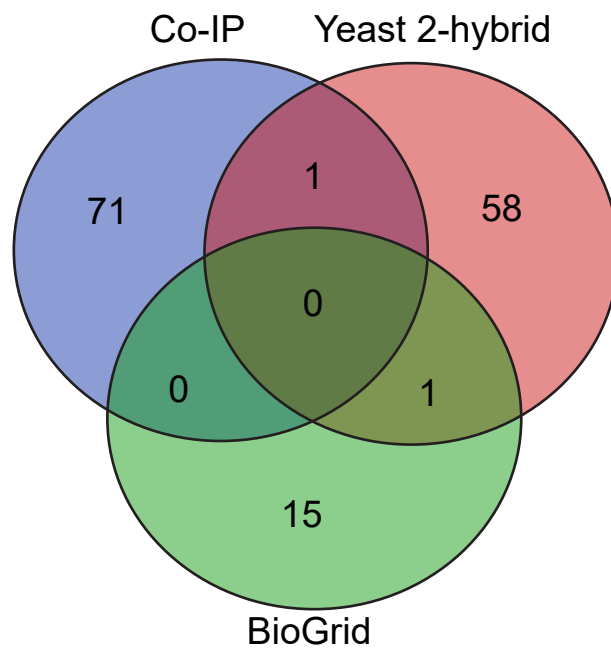


Figure S4: Venn diagram with the overlap between candidate interactor lists obtained by yeast 2-hybrid, Co-IP and from the BioGrid database of previously reported interactions.

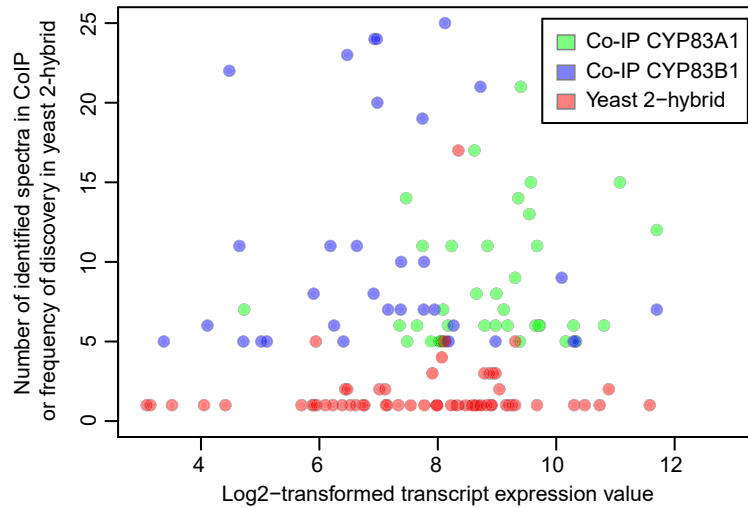


Figure S5: Number of identifications in yeast 2-hybrid screens and identified spectra in Co-IPs plotted against log₂-transformed average transcript expression values as a proxy for protein abundance levels. Average expression values of the respective transcripts in vegetative rosettes were retrieved from the eFP anatomy browser of the BAR database (Winter et al., 2007).

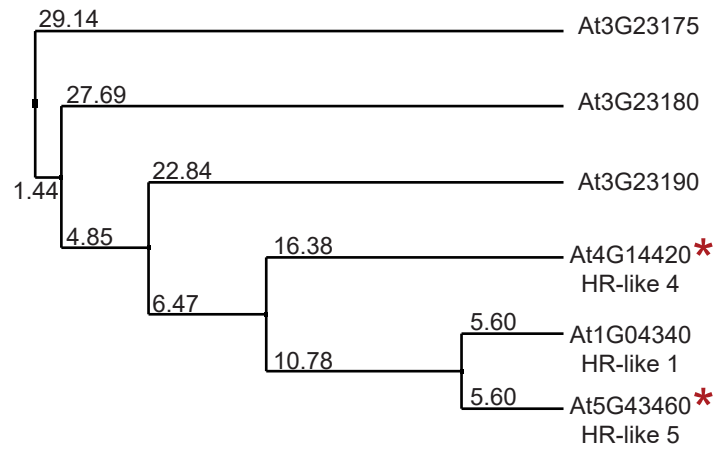


Figure S6: Phylogeny of six proteins annotated as HR-like lesion-inducing protein-related protein. Numbers represent distances based on neighbor joining by percentage identity. Proteins identified as interacting with glucosinolate biosynthetic proteins in yeast 2- hybrid screens are labeled with *.

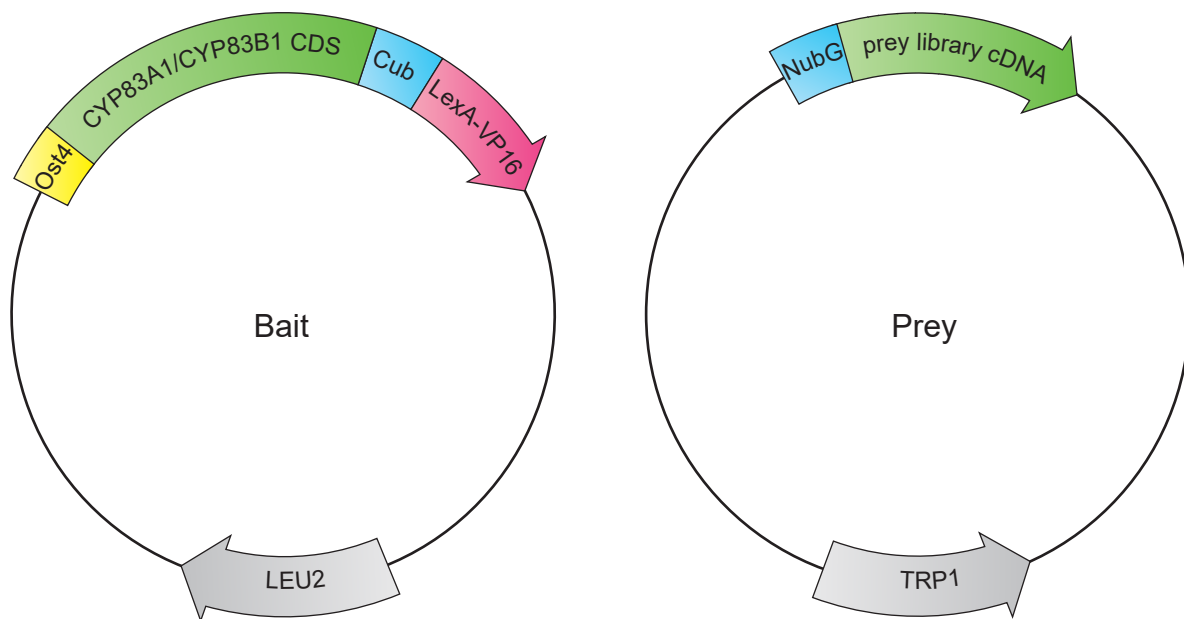


Figure S7: Schematic overview of bait and prey constructs used in yeast 2-hybrid screens. The bait vector encodes a fusion protein containing the Ost4-sequence to ensure correct integration into the yeast ER membrane, followed by the coding sequence (CDS) of either CYP83A1 or CYP83B1, the C-terminal half of ubiquitin (Cub) and the transcriptional activator LexA-VP16. The prey constructs contain the cDNA libraries generated from Arabidopsis mRNA and the sequence of a mutagenized N-terminal half of ubiquitin (NubG). The mutation prevents the ubiquitin halves from interacting intrinsically. The plasmids contain yeast selection markers (LEU2 and TRP1).

Upon protein-protein interaction, NubG and Cub reconstitute a functional ubiquitin, leading to proteolytic cleavage and release of LexA-VP16, and thereby the activation of the reporter system.

Table S1: Oligonucleotide sequences

| | |
|-------------------|---|
| 83A1_Sfi_F | ATTGAGGCCATTACGGCCATGGAAGATATCATCATCGGCG |
| 83B1_Sfi_F | ATTGAGGCCATTACGGCCATGGATCTCTTATTGATTATAGC |
| 83A1_Sfi_nostop_R | TCAATGGCCGAGGCGGCCAATACTTGTTCACTTTGTCTGG |
| 83B1_Sfi_nostop_R | TCAATGGCCGAGGCGGCCAGATGTGTTTCGTTGGTGAAGAAC |
| USER_F | GGGGCTGAGGTTTAATTAAGGATCCTTAATTAAGCCTCAGCCAAAAT |
| USER_R | GGGCTGAGGTTTAATTAAGGATCCTTAATTAAGCCTCAGCCCAAT |
| AT5G45420_FW | GGCTTAAUATGGATTTTTTCGACGAAGACAGG |
| AT5G51010_FW | GGCTTAAUATGGCGATGCAGCTTCC |
| AT5G52980_FW | GGCTTAAUATGGATCAACCCGACCCG |
| AT3G60600_FW | GGCTTAAUATGAGTAACATCGATCTGATTGGGA |
| AT5G42000_FW | GGCTTAAUATGTACGTAAGAGCACTTCCGA |
| AT2G33120_FW | GGCTTAAUATGGCGCAACAATCGTTGAT |
| AT2G41110_FW | GGCTTAAUATGGCGGATCAGCTCACA |
| AT5G61790_FW | GGCTTAAUATGAGACAACGGCAACTATTTTCC |
| AT2G32720_FW | GGCTTAAUATGGGAGACGAAGCAAAGATC |
| AT1G04750_FW | GGCTTAAUATGGCGCAACAATCGTTGA |
| AT2G28900_FW | GGCTTAAUATGCCTTCAAGCACATTCTCC |
| AT1G28490_FW | GGCTTAAUATGTCTTCAGCTCAAGATCCATT |
| AT4G14420_FW | GGCTTAAUATGGAGTTAGCTTCTTCT |
| AT5G43460_FW | GGCTTAAUATGGGTTTCTTCTTTTTCTTG |
| AT5G45420_REV | GGTTTAAUTTAATTAGCTGGAGTTTTCGAGCTC |
| AT5G51010_REV | GGTTTAAUTTAATTTTCATGAGGTGCTATTGACATAGAAG |
| AT5G52980_REV | GGTTTAAUTTAATTCTATTGATTTTTCTTAGTAGTGGGAGTGA |
| AT3G60600_REV | GGTTTAAUTTAATTTTATGTCCTCTTCATAATGTATCCCAAAT |
| AT5G42000_REV | GGTTTAAUTTAATTTTCATTGGTCTCCATTGATTCCAAAT |
| AT2G33120_REV | GGTTTAAUTTAATTTTATTTACCGAGTTGAATCCCC |
| AT2G41110_REV | GGTTTAAUTTAATTTCACTTAGCCATCATAACCTTCAC |
| AT5G61790_REV | GGTTTAAUTTAATTCTAATTATCACGTCTCGTTGCC |
| AT2G32720_REV | GGTTTAAUTTAATTCTACCCTGATTTGGTGTAGATACG |
| AT1G04750_REV | GGTTTAAUTTAATTTTAACTTAAACCCATGGCAAAT |
| AT2G28900_REV | GGTTTAAUTTAATTTTCAGTAGAAATAATGATTGTTAACGAACTGA |
| AT1G28490_REV | GGTTTAAUTTAATTTTAGGTCAAGAAGACAAGAACGAATAG |
| AT4G14420_REV | GGTTTTAAUCTAGGCAGCAGCTTTCGGGTTA |
| AT5G43460_REV | GGTTTTAAUTCAGTTTGCTTTTCCCTT |
| USER_XFP_F | GGCTTAAUTAAGGATCCTTAATTAACCTCAGCACGTGAGCAAGGGCGAGGAGCTG |
| USER_XFP_R | GGTTTAAUTTACTTGTACAGCTCGTCC |
| Pro_83A1_F | GGCTTAAUCTGTAAGAGAAACGAAATTCGG |
| Fus_Pro_83A1_R | ATTCTTAGTUGTTACTACTTTTGAGTGTTACATTG |
| Pro_83B1_F | GGCTTAAUAAGTTGGAGGAAGATTTGACAG |
| Fus_Pro_83B1_R | ATTTTTTTCUGTTTGACTTACTTAAGTCTTTG |
| Fus_83A1_F | AACATAAGAAUGGAAGATATCATCATCGG |
| 83A1_Nostop_R | GGTTTAAUATACTTGTTCACCTTCTCTGG |
| Fus_83B1_F | AGAAAAAAUUGGATCTCTTATTGATTATAGC |
| 83B1_Nostop_R | GGTTTAAUGATGTGTTTCGTTGGTGAAG |
| CYP83A1_FW | GGCTTAAUATGGAAGATATCATCATCGGCGTGG |
| CYP83A1_NSR | GGTTTAAUGGATACTTGTTCACCTTCTCTGGAACA |
| CYP83B1_FW | GGCTTAAUATGGATCTCTTATTGATTATAGCCGG |
| CYP83B1_NSR | GGTTTAAUGGGATGTGTTTCGTTGGTGAAG |
| At1G04340_FW | GGCTTAAUATGGGTTTCTTCTTTTTCTTG |
| At1G04340_NSR | GGTTTAAUCCAGCAGCTTTAGGCTTGGGAG |
| At4G14420_FW | GGCTTAAUATGGAGTTAGCTTCTTCT |
| At4G14420_NSR | GGTTTAAUGGGTTTGCTTTTGCCTTTGGAG |
| At5G43460_FW | GGCTTAAUATGGGTTTCTTCTTTTTCTTG |
| At5G43460_NSR | GGTTTAAUCCGGCAGCTTTCGGCTTAGGTG |

Table S2: Calculation of library transformation efficiencies. Dilutions of the four library transformations were plated on SD-LW medium and colony forming units were counted to determine the absolute number of transformants.

| Bait Library | CYP83A1 Hypocotyl | CYP83A1 Node | CYP83B1 Hypocotyl | CYP83B1 Node |
|---------------------|--------------------------|---------------------|--------------------------|---------------------|
| 1:100 | 50 | 164 | 58 | 142 |
| 1:1000 | 5 | | 6 | 9 |
| 1:10000 | 1 | 1 | 2 | 2 |
| Titer | 2.5×10^5 | 8.2×10^5 | 2.9×10^5 | 7.1×10^5 |

Table S3: T-DNA insertion lines used to assess impact of putative interactors on glucosinolate accumulation

| Gene | Insertion line | Name/Annotation |
|-----------|----------------|---|
| At1G04340 | SALK_062978C | HR-like lesion-inducing protein-related |
| At1G04750 | SALK_037273C | Vesicle associated membrane protein 721 (VAMP721); VAMP7B |
| At1G15500 | SALK_031126C | NTT2; ATP:ADP antiporter |
| At1G16890 | SALK_001977C | Ubiquitin-conjugating enzyme 36 (UBC36) |
| At1G26670 | SALK_116968C | Vesicle transport V-SNARE 12 (VTI12); VTI1B |
| At1G28250 | SALK_081490C | unknown |
| At1G28490 | SALK_107167C | SYNTAXIN OF PLANTS 61 (SYP61); OSM1 |
| At1G30935 | SALK_084201C | F-box protein family pseudogene |
| At1G44920 | SALK_137362C | unknown |
| At1G52870 | SALK_133672C | Mpv17/PMP22 family; Peroxisomal membrane protein |
| At1G57765 | SALK_103508C | unknown |
| At1G59890 | SALK_043513C | SIN3-LIKE 5, SNL5 |
| At1G66240 | SALK_021013C | Homolog of anti-oxidant 1 (ATX1) |
| At2G05630 | SALK_145672C | ATG8D |
| At2G32380 | SALK_079608C | Transmembrane protein 97 |
| At2G32720 | SALK_009185C | Cytochrome B5-B (CB5-B) |
| At2G33120 | SALK_103189C | Vesicle associated membrane protein 722 (VAMP722); Synaprobrevin-related protein 1 (SAR1) |
| At2G41110 | SALK_089283C | CALMODULIN 2 (CAM2); ATCAL5 |
| At3G48990 | SALK_057339C | Acyl-activating enzyme 3 (AAE3) |
| At3G50685 | SALK_133139C | unknown |
| At3G60600 | SALK_021679C | VAMP/Synaprobrevin-associated protein 27-1 (VAP27, VAP27-1), Vesicle associated protein |
| At4G14420 | SALK_008498C | HR-like lesion-inducing protein-related |
| At4G24920 | SALK_021134C | Sec61 subcomplex, putative γ subunit; P-P-bond-hydrolysis-driven protein transmembrane transporter |
| At4G27500 | SALK_009375C | Proton pump interactor 1 (PPI1) |
| At4G30950 | SALK_000835C | Fatty acid desaturase 6 (FAD6); FADC; SFD4; Stearoyl desaturase deficiency 4 |
| At5G16830 | SALK_054274C | Syntaxin of plants 21 (SYP21); PEP12 |
| At5G20130 | SALK_058047C | unknown |
| At5G24680 | SALK_051987C | Peptidase C78, ubiquitin fold modifier-specific peptidase 1/ 2 |
| At5G42570 | SALK_142314C | B-cell receptor-associated 31-like |
| At5G43460 | SALK_030116C | HR-like lesion-inducing protein-related |
| At5G43580 | SALK_057639C | Unusual serine protease inhibitor (UPI) |
| At5G52240 | SALK_035827C | Membrane-associated progesterone binding protein 5 (MAPR5); ATMP1; Membrane steroid binding protein 1 (MSBP1) |
| At5G60920 | SALK_106466C | COBRA (COB) |
| At5G61790 | SALK_083600C | Calnexin 1 (CNX1) |
| At5G67600 | SALK_038916C | WIH1 (WINDHOSE 1) |

Table S4: Two-way ANOVA testing for effects of genotype and plant tray on aliphatic and indole glucosinolates with the linear model aliphatic or indole glucosinolates = Genotype + Tray.

| | | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|--------------------------|-----------|-----|--------|----------|---------|-------------|
| Aliphatic glucosinolates | Genotype | 34 | 1782 | 0.05241 | 4038 | 1.17e-11*** |
| | Tray | 1 | 0.001 | 0.00109 | 0.084 | 0.772 |
| | Residuals | 348 | 4517 | 0.01298 | | |
| Indole glucosinolates | Genotype | 34 | 0.1516 | 0.004460 | 1100 | 0.326 |
| | Tray | 1 | 0.0005 | 0.000518 | 0.128 | 0.721 |
| | Residuals | 348 | 14106 | 0.004053 | | |

*** indicates $p < 0.001$

Analyzed genotypes are wildtype (n = 22) or T-DNA insertion lines (n = 6-12) listed in Table S3.

Aliphatic glucosinolates:

3-methylthiopropyl-, 3-methylsulfinyl-, 4-methylthiobutyl-, 4-methylsulfinylbutyl-, 5-methylsulfinylpentyl-, 7-methylthioheptyl-, 7-methylsulfinylheptyl-, 8-methylthiooctyl-, 8-methylsulfinyloctyl-glucosinolate

Indole glucosinolates:

Indol-3-ylmethyl-, 4-methoxy-indol-3-ylmethyl-, N-methoxy-indol-3-ylmethyl-glucosinolate

Table S5: Two-way ANOVA testing for effects of T-DNA insertions on different classes of glucosinolates with the linear model short-chained aliphatic, long-chained aliphatic, total aliphatic or indole glucosinolates = Genotype + Experiment.

| | | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|--|------------|-----|--------|---------|---------|-------------|
| Short-chained aliphatic glucosinolates | Genotype | 7 | 22.48 | 3.211 | 25.13 | <2e-16*** |
| | Experiment | 1 | 4.16 | 4.156 | 32.53 | 2.44e-08*** |
| | Residuals | 363 | 46.38 | 0.128 | | |
| Long-chained aliphatic glucosinolates | Genotype | 7 | 0.42 | 0.0596 | 0.39 | 0.908 |
| | Experiment | 1 | 0.40 | 0.4003 | 2.62 | 0.106 |
| | Residuals | 363 | 55.45 | 0.1528 | | |
| Aliphatic glucosinolates | Genotype | 7 | 8.16 | 11.660 | 11.14 | 8.83e-13*** |
| | Experiment | 1 | 1.63 | 16.276 | 15.55 | 9.65e-05*** |
| | Residuals | 363 | 38.00 | 0.1047 | | |
| Indole glucosinolates | Genotype | 7 | 1.56 | 0.2228 | 1.657 | 0.1184 |
| | Experiment | 1 | 0.46 | 0.4635 | 3.448 | 0.0641 |
| | Residuals | 363 | 48.80 | 0.1344 | | |

*** indicates $p < 0.001$

Analyzed genotypes are wildtype or single (*hr1*, *hr4*, *hr5*), double (*hr1/4*, *hr1/5*, *hr4/5*) or triple (*hr1/4/5*) T-DNA insertion lines.

n = 43-48 for each genotype in two independent experiments with n = 21-24 each.

Short-chained aliphatic glucosinolates:

3-methylthiopropyl-, 3-methylsulfinyl-, 3-hydroxypropyl-, 3-benzoyloxypropyl-, 4-methylthiobutyl-, 4-methylsulfinylbutyl-, 4-hydroxybutyl-, 4-benzoyloxybutyl-, 5-methylsulfinylpentyl-glucosinolate

Long-chained aliphatic glucosinolates:

7-methylthioheptyl-, 7-methylsulfinylheptyl-, 8-methylthiooctyl-, 8-methylsulfinyloctyl-glucosinolate

Aliphatic glucosinolates:

Short-chained aliphatic and long-chained aliphatic glucosinolates

Indole glucosinolates:

Indol-3-ylmethyl-, 4-methoxy-indol-3-ylmethyl-, N-methoxy-indol-3-ylmethyl-glucosinolate

Table S6: Interactors found in the BioGrid database

| Gene | Name/Annotation | Interactor of |
|-----------|---|---------------|
| At1G14700 | Purple acid phosphatase 3 (PAP3) | CYP83A1 |
| At1G17280 | Ubiquitin-conjugating enzyme 34 (UBC34) | CYP83A1 |
| At1G21240 | Wall-associated kinase 3 (WAK3) | CYP83A1 |
| At1G29060 | Golgi localized QcSNARE involved in response to salt and osmotic stress | CYP83A1 |
| At2G26180 | IQ-domain 6 (IQD6) | CYP83A1 |
| At3G05120 | GA insensitive dwarf 1A (GID1A) | CYP83B1 |
| At3G08510 | Phosphoinositide-specific phospholipase C (PI-PLC) | CYP83A1 |
| At3G10640 | VPS60.1 | CYP83A1 |
| At3G12180 | Cornichon family protein | CYP83A1 |
| At3G48890 | Membrane steroid binding protein 2 (MSBP2) | CYP83A1 |
| At4G20790 | Leucine-rich repeat protein kinase family protein | CYP83A1 |
| At4G27610 | unknown | CYP83A1 |
| At4G35950 | GTP binding protein, member of ROP GTPases gene family-like (RAC6) | CYP83A1 |
| At5G47120 | Bax inhibitor 1, functions as an attenuator of cell death (BI-1) | CYP83A1 |
| At5G52240 | Membrane steroid binding protein 1 (MSBP1) | CYP83A1 |
| At5G59650 | Leucine-rich repeat protein kinase family protein | CYP83A1 |