New Phytologist Supporting Information

Article Title: Resistance to a non-selective HPPD-inhibiting herbicide via novel reduction-dehydration-glutathione conjugation in Amaranthus tuberculatus

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**Table S1.** Mass spectral fragment ions and their relative abundance of a non-commercial, non-selective, HPPD-inhibiting herbicide, syncarpic acid-3 (SA3). \( m/z \) [M-H]—refers to the mass-to-charge ratio of the compound detected using LC-MS negative electrospray ionization mode.

| Ion # | \( m/z \) [M-H] | Ion abundance area | % of major ion |
|-------|-----------------|-------------------|----------------|
| 1     | 421.0875        | 290729056         | 100.0000       |
| 2     | 213.0147        | 31250244          | 10.7489        |
| 3     | 281.0050        | 7684854           | 2.6433         |
| 4     | 165.0175        | 3120191           | 1.0732         |
| 5     | 257.0042        | 2622193           | 0.9019         |
| 6     | 196.0363        | 1959112           | 0.6739         |
| 7     | 137.0235        | 1906981           | 0.6559         |
| 8     | 185.0214        | 1258195           | 0.4328         |
| 9     | 244.4986        | 1226286           | 0.4218         |
| 10    | 264.8076        | 1169516           | 0.4023         |
| 11    | 124.4851        | 1152218           | 0.3963         |
| 12    | 71.7762         | 1141201           | 0.3925         |
| 13    | 310.9516        | 1133761           | 0.3900         |
| 14    | 179.5400        | 1107058           | 0.3808         |
| 15    | 108.0269        | 1059604           | 0.3645         |
| 16    | 90.1439         | 1028508           | 0.3538         |
| 17    | 92.5667         | 974687            | 0.3353         |
| 18    | 55.4568         | 919609            | 0.3163         |
| 19    | 51.3630         | 871016            | 0.2996         |
| 20    | 61.5124         | 866217            | 0.2979         |
Table S2. Ten most discriminating compounds for syncarpic acid-3 (SA3)-treated excised leaf samples.

| Compound code | Molecular Weight | Retention Time (min) | Dimension 1 | Dimension 2 |
|---------------|------------------|----------------------|-------------|-------------|
| ELA_1877      | 422.096          | 22.29                | 0.146893    | 0.002758    |
| ELA_1830      | 408.080          | 20.83                | 0.138833    | -0.00081    |
| ELA_1929      | 438.091          | 20.50                | 0.131861    | 8.32E-05    |
| ELA_0816      | 214.021          | 18.37                | 0.119650    | 0.01337     |
| ELA_2343      | 582.097          | 18.38                | 0.107778    | -0.02676    |
| ELA_2513      | 713.149          | 16.18                | 0.084446    | -0.03008    |
| ELA_2079      | 472.268          | 18.83                | 0.061091    | -0.03984    |
| ELA_0799      | 210.956          | 3.67                 | 0.053954    | 0.000464    |
| ELA_1909      | 430.257          | 18.58                | 0.051125    | -0.02272    |
| ELA_2615      | 972.495          | 15.77                | 0.046898    | -0.00709    |
Figure S1. Standard curve of syncarpic acid-3 (SA3) for quantifying remaining SA3 parent in excised leaves of four waterhemp populations. Peak identification and abundance were acquired using a high-performance liquid chromatography with a photodiode array detector at 280 nm.
**Figure S2.** Flow diagram of metabolite profiling carried out on waterhemp populations treated with the non-selective, HPPD-inhibiting herbicide, syncarpic acid-3 (SA3).
Figure S3. First generation product ion (MS2) mass spectra of syncarpic acid-3 (SA3).

IUPAC name: 6-[2,4-bis(trifluoromethyl)benzoyl]-2,2,4,4-tetramethylcyclohexane-1,3,5-trione, Molecular formula: C_{19}H_{16}F_{6}O_{4}; average mass: 422.3183592 g/mol; exact mass: 421.08701 Da [M-H]; experimental mass: 421.0927 Da [M-H]; corrected experimental mass: 421.0865 Da [M-H]. The fragmentation patterns of the major product ions of SA3 (m/z 213 [M-H], m/z 196 [M-H] and m/z 257 [M-H+O]) are shown on the structure. Carbon atoms are numbered as 1-10 in the cyclohexane ring and 1’-9’ in the benzoyl ring, respectively. Data was acquired using ultra-performance liquid chromatography-tandem mass spectrometry in the negative electrospray ionization mode.
**Figure S4.** Orthogonal partial least squares discriminant analysis (OPLS-DA) of the metabolites from two waterhemp populations: (1) multiple-herbicide-resistant population from Stanford, Illinois, USA (SIR) and (2) 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor-sensitive population from Adams County, Illinois, USA (ACR), treated with 0.3 mM syncarpic acid-3 (SA3). (a) Scores are labeled according to population and colored according to treatment; (b) hierarchical cluster analysis (HCA) dendrogram of the hierarchical clustering of metabolites in these samples; (c) loadings scatter plot of metabolites detected using an ultra-performance liquid chromatography-tandem mass spectrometer (Thermo Scientific™). Loadings are colored based on the group assigned after HCA using the Manhattan distance function and Ward linkage method.
Figure S5. Possible, but not detected, structures of fragment ions derived from metabolism of the aryl ring of syncarpic acid-3 (SA3). These proposed fragment ions were not detected in the mass spectra of the metabolite features that were discriminating for SA3-treated samples.
Figure S6. Correlation plot of the putative metabolites of syncarpic acid-3 (SA3) detected in leaf extracts of waterhemp populations SEN, ACR, NEB and SIR. Plants were treated with 0.3 mM SA3. SA3 is negatively correlated with all the putative metabolites except for M438. Strong Pearson’s $r$ values are exhibited between the putative dehydrated-SA3 and the proposed Phase II metabolites, M527, M582, M584 and M713.
Figure S7. Chemical structures of herbicides detoxified in plants via GST-catalyzed glutathione conjugation. (a) 2-chloro-4-ethylamino-6-isopropylamino-s-triazine (atrazine) (Shimabukuro, 1967; Lamoureux et al., 1970; Shimabukuro et al., 1970), and (b) 2-chloro-N-(2-ethyl-6-methylphenyl)-N-[(2S)-1-methoxypropan-2-yl]acetamide (S-metolachlor) (O'Connell et al., 1988). Glutathione conjugation sites (i.e., electrophilic carbons) in each compound are indicated by a blue arrow.
Figure S8. Chemical structures of Michael acceptors (a) E9 and (b) E10 (Mayer & Ofial, 2019) resembling the putative electrophilic Phase I metabolite of syncarpic acid-3, (c) M406.
Methods S1  LC-MS, data processing and statistical analysis of metabolomics data

Liquid chromatography-mass spectrometry

Untargeted metabolomics of waterhemp leaf extracts was carried out using a Dionex Ultimate 3000 series HPLC system (Thermo Scientific™, Germering, Germany) coupled to a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific™, Bremen, Germany) as described previously (Elolimy et al., 2019). The chromatographic analysis was conducted in a randomized sequence order with quality control (QC) samples in order to equilibrate the analytical platform. QC samples were prepared by mixing aliquots of all test samples (Sangster et al., 2006; Dunn et al., 2011; Godzien et al., 2015) and were injected after every 10 test samples to evaluate the stability of the experimental procedure (Wehrens et al., 2016).

The chromatographic analysis was carried out in a randomized sequence order with five injections of pure methanol at the beginning of the sequence for apparatus equilibration, followed by one injection of the sample solvent (water:acetonitrile, 1:1, v/v), and three injections of the quality control (QC) samples in order to equilibrate the analytical platform. QC samples were prepared by mixing aliquots of all test samples (Sangster et al., 2006; Dunn et al., 2011; Godzien et al., 2015) and were injected after every 10 test samples to evaluate the stability of the experimental procedure (Wehrens et al., 2016).

Data pre-processing

Raw data files obtained in full-MS mode (samples, procedural blank, and QC) and data obtained in full-MS followed by data-dependent MS2 were processed by Compound Discoverer™ software version 3.1.305 (Thermo Scientific™, Bremen, Germany) for initial data processing.
including peak detection, peak alignment and peak integration. Briefly, raw files were aligned with adaptive curve setting with 5 ppm mass tolerance and 0.2 min retention time shift. Peaks with less than a two-fold increase, compared to blank samples, and those detected in less than 50% of QC samples, and where the relative standard deviation (%RSD) of the QC samples was greater than 30%, were removed from the list. Unknown compounds, detected with a five ppm mass tolerance, three signal-to-noise ratio, 30% of relative intensity tolerance for isotope search and 500,000 minimum peak intensity, were grouped with five ppm mass and 1 min retention time tolerances.

Since the ESI (-) had increased peak abundance of putative SA3 metabolites compared to the ESI (+) mode, a separate data analysis was conducted on the ESI (-) data. Raw data (.RAW) from the single quadrupole instrument was converted to .mzML format with the MSConvertGUI from ProteoWizard (http://proteowizard.sourceforge.net/tools.shtml). MS-DIAL v. 4.24 (with open source publicly available EI spectra library), was used for extraction of raw peaks, baseline filtering and calibration, peak alignment, deconvolution, peak identification, peak normalization, and integration of the peak height following the parameters as previously described (Tsugawa et al., 2015). An average peak width of 20 scans and a minimum peak height of 500,000 amplitudes was applied for peak detection, and a sigma window value of 0.5, EI spectra cut-off of 5,000 amplitudes was implemented for deconvolution. The putative SA3 metabolites, all unknowns (Table 2) (Sumner et al., 2007), were inspected for MS2 fragmentation patterns and normalized peak areas were exported for comparisons among waterhemp populations.
Statistical analysis of metabolomics data

Prior to multivariate statistics, a compound detection rate of 50% relative to the QC samples was set (Broadhurst et al., 2018), leaving a total of 2,580 and 2,930 compounds for the excised leaf assay and the whole plant assay samples, respectively. For multivariate analysis, the peak areas of metabolite features of waterhemp leaf extracts were normalized via log transformation. Peak areas were then Pareto scaled to moderate the influence of compounds produced at high concentrations and to recognize the effects of compounds produced at relatively lower concentrations in the overall characterization of waterhemp metabolites (van den Berg et al., 2006). To visualize trends in the global metabolomic data between the waterhemp populations, data were represented by unsupervised modelling through principal components analysis (PCA) using SIMCA v15 (Umetrics, Sweden). Next, to determine the discriminating compounds in the SA3-treated samples used in this study, orthogonal projections to latent structures discriminant analysis (OPLS-DA) was conducted, again in SIMCA v15 (Umetrics, Sweden). OPLS-DA maximizes the covariance of the matrix of predictor data, X (metabolite features), and the response block, Y (treatment), thus providing a more powerful prediction of among-group variation compared to PCA (Worley et al., 2013). Following this analysis, hierarchical clustering using Ward’s linkage method was carried out on the X variables. Pearson’s correlation analysis and generation of correlation plots were performed for putative SA3 metabolites using the “corrplot” package in R studio (R Core Team, 2013). Visual comparisons of the relative abundance of putative SA3-metabolites were carried out using GraphPad Prism 8 software (GraphPad Software Inc, San Diego, CA, USA).
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