**Data S1. Identification of *M. truncatula* isoflavonoids.**

Based on the PCA analysis presented in Figure 2f, a subset of 124 *m*/*z* peaks with a higher abundance in the EMA1OE lines were considered as important contributors to the difference between the EMA1OE and the control lines. Peak grouping (based on retention time) revealed that 61 of those peaks were attributed to only two metabolites, eluting at 14.68 and 16.59 min, respectively, with a dramatically increased abundance in the EMA1OE lines. Upon inspection of the LC-ESI-FT-ICR-MS chromatograms, these peaks appear visibly increased in the EMA1OE lines (Figure 1a of Data S1). The MS scan of the first peak, eluting at 14.68 min, contained a [2M‑H]- dimer ion with an accurate mass of 1031.24517 Da. A minor [M‑H]- peak appeared at *m*/*z* 515.12026, corresponding to the brutoformula C25H24O12 (δppm = 1.477). An additional in-source fragment appeared at *m*/*z* 267.06640 (C16H12O4; δppm = 0.441; Figure 1b of Data S1). Similarly, the peak eluting at 16.59 min has an [M-H]- ion at *m*/*z* 517.13644 (C25H26O12; δppm = 2.496) and an in-source fragment at *m*/*z* 269.08229 (C16H14O4; δppm = 1.330; Figure 1b of Data S1).



**Figure 1.** LC-ESI-FT-ICR-MS analysis of CTR and EMA1OE lines. (a) LC-ESI-FT-ICR-MS chromatograms of an extract from CTR (black) and EMA1OE (red) roots. The peaks at *t*R 14.68 and 16.59 min are dramatically increased in the EMA1OE lines. (b) MS scans of the peaks at *t*R 14.68 (left) and 16.59 (right) min.

The metabolite eluting at 14.68 was further elucidated based on its MS*n* fragmentation. The MS2 fragmentation of the dimer at *m*/*z* 1031.25 led to major daughter ions at *m*/*z* 987, 943, 781, 739, 515, and 471 (Figure 2a of Data S1), with the daughter ion at *m*/*z* 515 corresponding to the [M‑H]- ion. The daughter ion at *m*/*z* 471 results from a neutral loss of 44 Da from the [M‑H]- ion, corresponding to the loss of a CO2 moiety. Indeed, the ion at *m*/*z* 471 also occurs as an in-source fragment of the unknown metabolite and has an accurate mass of 471.13036 Da (Figure 2b of Data S1), corresponding to the brutoformula C24H24O10 (δppm = 1.464), which again indicates the loss of a CO2 moiety from the unknown metabolite with a calculated brutoformula of C25H24O12. Fragmentation of the in-source fragment ion at *m*/*z* 471 led to a single daughter ion at *m*/*z* 267 (Figure 2c of Data S1), which corresponds to the in-source fragment ion with a calculated brutoformula of C16H12O4, implying a neutral loss of 204 Da or C8H12O6, corresponding to the loss of a hexose (162 Da; C6H10O5) and a ketene (42 Da; C2H2O). The loss of a CO2 and ketene moiety indicates the unknown metabolite likely corresponds to a malonylated compound, which further contains a hexose moiety and an aglycone with a calculated brutoformula of C16H12O4. Aglycones with this molecular formula include the isoflavone formononetin (Farag *et al.*, 2007). Indeed, the MS2 fragmentation of the aglycone at *m*/*z* 267 led to a single daughter ion at *m*/*z* 252 (Figure 2d of Data S1), indicating a loss of a methyl radical, typical of an *O*-methylated metabolite like formononetin. Furthermore, the MS3 fragmentation of the *m*/*z* 252 radical ion of the aglycone (Figure 2e of Data S1) corresponds well with the reported MS3 fragmentation of formononetin (Kang *et al.*, 2007). Taken together, the metabolite eluting at 14.68 min likely corresponds to formononetin glucoside malate (FGM), a metabolite known to accumulate in *M. truncatula* (Farag *et al.*, 2007).



**Figure 2.** Identification of FGM (formononetin glucoside malate) by MS*n* fragmentation. (a) MS2 fragmentation of the dimer at *m*/*z* 1031.25. (b) FT-MS scan revealing the accurate mass of the in-source fragment ion at *m*/*z* 471. (c) MS2 fragmentation of the in-source fragment ion at *m*/*z* 471. (d) MS2 fragmentation of the aglycone at *m*/*z* 267. (e) MS3 fragmentation of the *m*/*z* 252 radical ion of the aglycone at *m*/*z* 267.

Similarly, the metabolite eluting at 16.59 min was further elucidated based on its MS*n* fragmentation. MS2 fragmentation of the dimer at *m*/*z* 1035.28 led to major daughter ions at *m*/*z* 991, 947, 517, and 473 (Figure 3a of Data S1), with the daughter ion at *m*/*z* 517 corresponding to the [M‑H]- ion. The daughter ion at *m*/*z* 473 results from a neutral loss of 44 Da from the [M‑H]- ion, corresponding to the loss of a CO2 moiety. The ion at *m*/*z* 473 also occurs as an in-source fragment of the unknown metabolite and has an accurate mass of 473.14631 Da (Figure 3b of Data S1), corresponding to the brutoformula C24H26O10 (δppm = 2.092), which again indicates the loss of a CO2 moiety from the unknown metabolite with an accurate mass of 517.13620 Da (Figure 3b of Data S1) and a calculated brutoformula of C25H26O12 (δppm = 2.032). Fragmentation of the in-source fragment ion at *m*/*z* 473 led to a single daughter ion at *m*/*z* 269 (Figure 3c of Data S1). The loss of a CO2 moiety and a neutral loss of 204 Da is similar to the losses observed for FGM, implying a similar glucoside malate, but a different aglycone for this metabolite. The aglycone occurs as an in-source fragment ion with an accurate mass of 269.08229 (Figure 1b of Data S1) and a calculated brutoformula of C16H14O4 (δppm = 1.330). Aglycones with this molecular formula include the pterocarpan medicarpin, the isoflavone genistein and the flavanone naringenin (Farag *et al.*, 2007). The MS3 fragmentation of the daughter ion at *m*/*z* 269 leads to a single granddaughter ion at *m*/*z* 254 (Figure 3d of Data S1), indicating the aglycone is an *O*-methylated metabolite like medicarpin. Taken together, the metabolite eluting at 16.59 min likely corresponds to medicarpin glucoside malate (MGM), also a compound known to accumulate in *M. truncatula* (Farag *et al.*, 2007).



**Figure 3.** Identification of MGM (medicarpin glucoside malate) by MS*n* fragmentation. (a) MS2 fragmentation of the dimer at *m*/*z* 1035.28. (b) FT-MS scan revealing the accurate mass of the in-source fragment ion at *m*/*z* 473. (c) MS2 fragmentation of the in-source fragment ion at *m*/*z* 473. (d) MS3 fragmentation of the daughter ion at *m*/*z* 269.

Next to the 61 *m*/*z* peaks derived from FGM and MGM, 63 additional *m*/*z* peaks with a higher abundance in the EMA1OE lines were considered as important contributors to the difference between the EMA1OE and the control lines. Peak grouping revealed several lower abundant metabolites with at least two *m*/*z* peaks that significantly contribute to the difference between the EMA1OE and the control lines. One of these peaks, eluting at 14.92 min, has a [2M‑H]- dimer ion with an accurate mass of 1091.26436 Da (Figure 4a,b of Data S1). A minor [M‑H]- peak appeared at *m*/*z* 545.13014, corresponding to the brutoformula C26H26O13 (δppm = 0.140). Like for FGM and MGM, the metabolite is a glucoside malate, but with an aglycone in-source fragment ion with an accurate mass of 297.07681 (C17H14O5; δppm = ‑0.124; Figure 4b of Data S1). Aglycones with this molecular formula include the isoflavone afrormosin (Farag *et al.*, 2007). Indeed, the MS2 fragmentation of this aglycone (Figure 4c of Data S1) indicates the presence of an *O*-methyl group, and the loss of a second methyl radical in the MS3 fragmentation of the aglycone (282 🡪 267; Figure 4d of Data S1) implies the presence of a second *O*-methyl group, a fragmentation pattern that fits well with afrormosin as aglycone. Taken together, the metabolite eluting at 14.92 min likely corresponds to afrormosin glucoside malate (AGM), another compound known to accumulate in *M. truncatula* (Farag *et al.*, 2007).

Other *m*/*z* peaks with an apparent higher abundance in the EMA1OE lines were not identified as they either were the result of integration issues or too low abundant for MS*n* fragmentation and tentative identification.



**Figure 4.** Identification of AGM (afrormosin glucoside malate) by MS*n* fragmentation. (a) Normalized ion current of AGM in three control (CTR) and three EMA1OE (OE) lines showing higher abundance of AGM in the EMA1OE lines. Error bars indicate SEM; n = 5 technical replicates. Statistical significance was determined by a Student’s *t*-test with Welch correction (\*\*\*p<0.001) (b) FT-MS scan of the AGM peak eluting at 14.92 min. (c) MS2 fragmentation of the dimer at *m*/*z* 1091.26. (d) MS2 fragmentation of the in-source fragment at *m*/*z* 297.08. (e) MS3 fragmentation of the daughter ion at *m*/*z* 282. (f) MS4 fragmentation of the granddaughter ion at *m*/*z* 254.

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