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Comparative Metabolomic Analyses of *Ipomoea lacunosa* Biotypes with Contrasting Glyphosate Tolerance Captures Herbicide-Induced Differential Perturbations in Cellular Physiology

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Supporting Information

ABSTRACT: Glyphosate-tolerant *Ipomoea lacunosa* is emerging as a problematic weed in the southeastern United States. Metabolomic profiling was conducted to examine the innate physiology and the glyphosate induced perturbations in two biotypes of *I. lacunosa* (WAS and QUI) that had contrasting glyphosate tolerance. Compared to the less tolerant QUI-biotype, the innate metabolism of the more tolerant WAS-biotype was characterized by a higher abundance of amino acids, and pyruvate; whereas the sugar profile of the QUI biotype was dominated by the transport sugar sucrose. Glyphosate application (80 g ae/ha) caused similar shikimate accumulation in both biotypes. Compared to QUI, in WAS, the content of aromatic amino acids was less affected by glyphosate treatment, and the content of Ala, Val, Ile, and Pro increased. However, the total sugars decreased by ∼75% in WAS, compared to ∼50% decrease in QUI. The innate, higher proportional abundance, of the transport-sugar sucrose in QUI coud partly explain the higher translocation and greater sensitivity of this biotype to glyphosate. The decrease in sugars, accompanied by an increase in amino acids could delay feedback regulation of upstream enzymes of the shikimate acid pathway in WAS, which could contribute to a greater glyphosate tolerance. Our study, through a metabolomics approach, provides complementary data that elucidates the cellular physiology of herbicide tolerance in *Ipomoea lacunosa* biotypes.

KEYWORDS: *glyphosate tolerance, Ipomoea lacunosa, nontargeted metabolomics, pathway analysis, pitted morningglory*

INTRODUCTION

Since the introduction of glyphosate-resistant (GR) crops, the herbicide glyphosate has been used extensively in agriculture worldwide for weed management.[1] Consequently, the over-reliance on glyphosate for weed management in GR cropping systems has resulted in the evolution of glyphosate resistance in 37 weed species.[2] Moreover, several weed species, including species of the genera Ipomoea, also have varying degrees of natural tolerance to glyphosate.[3] Among Ipomoea spp., *I. lacunosa* (pitted morningglory) is one of the most common and troublesome weed species in southern United States row crop production systems.[4,5] Over the years, this species has risen to be one of the most common and difficult to control weeds in cotton (*Gossypium hirsutum* L.) and soybean (*Glycine max* (L.) Merr.), especially in 11 southern states.[5,6] Being glyphosate tolerant, the efficacy of glyphosate to control *I. lacunosa* is often variable with several studies reporting inadequate control at the field-recommended rates (0.84−1.26 kg ae ha^{-1}).[7−9] Reduced susceptibility of this species to glyphosate is generally attributed to limited foliar absorption[7] or reduced translocation of the herbicide from the treated leaves to the target sites.[10] However, other studies have reported a minimal role of differential absorption and translocation in conferring glyphosate tolerance in *I. lacunosa*.[11] Thus, there is a critical knowledge gap in relating the differential glyphosate tolerance of *I. lacunosa* biotypes with their respective metabolic processes.

Metabolomics is an emerging field which provides a finer understanding of the functioning of physiological systems by quantifying small intermediary molecules (metabolites) within the dynamic framework of the metabolome.[12] Significant advances in analytical methods, data capture and analysis have resulted in an improved sensitivity, accuracy, and capacity.[13] Unlike genomics, transcriptomics, and proteomics where the respective molecular markers are susceptible to biological alterations and functional modifications, metabolomics offers unique approaches to understand the biological information associated with the metabolites. Targeted and nontargeted metabolite profiling can be considered as a complementation to other biomolecular profiling technologies to explain the physiological mechanisms that contribute to differential cellular physiology.[14,15] Previously, metabolomics have been employed to assess glyphosate effects in peas roots and crop-beneficial *Pseudomonas* species.[16,17] Despite this advantage, very few studies have employed metabolomics to better understand the physiological influences of herbicides on weeds.[18,19] Through metabolomics approach, it was shown that the glyphosate resistant biotypes of *Amaranthus palmeri*

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initially (8 h after application) exhibit similar herbicide-induced metabolic perturbations as that of the susceptible biotypes, but recover by 80 h after treatment.[19] Therefore, understanding the cellular physiology of herbicidal effects might enable knowledge-based adoption of alternate herbicides and cultural practices that may specifically target the metabolic vulnerabilities of resistant or tolerant biotypes. Also, a metabolomics approach could differentiate weedy biotypes with varied susceptibility to herbicides, based on their native cellular metabolism in the absence of herbicide stress.

The present study aims to characterize the innate differences (differences in the absence of glyphosate stress) in cellular physiology and the glyphosate-induced physiological perturbations in two biotypes of *I. lacunosa* that exhibit contrasting levels of natural tolerance to glyphosate using nontargeted metabolite profiling. In contrast to targeted profiling, where the identification is limited to metabolites of interest, nontargeted profiling methods would achieve the widest possible metabolic coverage by detecting chemically diverse metabolites with varying abundance. We hypothesized that (i) the differential sensitivity of *I. lacunosa* biotypes to glyphosate could be partially explained by innate differences in their metabolism, (ii) apart from the disruption of aromatic amino acids, the glyphosate would induce systemic cellular perturbations that alter other metabolic pathways, especially in the more sensitive biotype.

MATERIALS AND METHODS

Plant Biotypes. Seeds of glyphosate-tolerant *I. lacunosa* biotypes were obtained from Stoneville, MS (Crop Production Systems Research Unit, USDA-ARS, Stoneville, MS). These biotypes have been previously characterized as most tolerant (MS-WAS-8 (WAS); GR_{50} 151.4 g ae ha^{-1}) and least tolerant (MS-QUI-1 (QUI); GR_{50} 59.1 g ae ha^{-1}).[10] Seeds were planted in individual pots (10 cm diameter x 9 cm deep) containing commercial germination mixture (Sun-Gro Redi-Earth Plug and Seedling Mix, Sun-Gro Horticulture, Bellevue, WA 98008), and following the emergence of two true leaves, the plants were fertilized with 50 mL of 4 g L^{-1} fertilizer (MiracleGrow, 24%−8%−16%, Scotts Miracle-Gro Products, Inc., Marysville, OH). The plants were grown in a greenhouse maintained at day/night temperatures of 30/20 °C, respectively, with a 14 h photoperiod and subirrigated every alternate day until harvest.

Experimental Design and Glyphosate Application. Plants of uniform growth (height and leaf numbers) from each biotype were selected and randomly assigned to two treatment groups: water (control) and glyphosate. Two weeks after planting, respective treatments (water or glyphosate) were applied to five plants per biotype using an

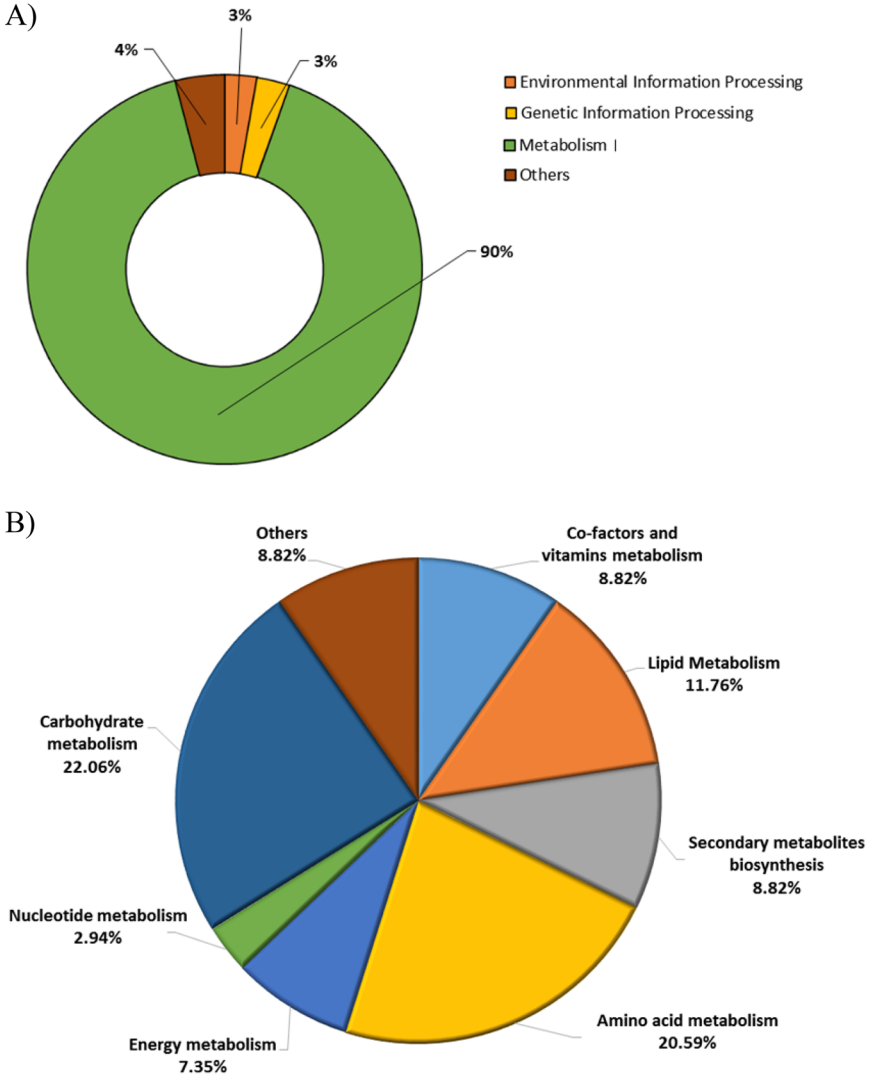

Figure 1. Ontological classification of metabolites identified in leaves of water-treated WAS and QUI biotypes of *Ipomoea lacunosa* harvested 80HAT. Panel (A) depicts the broad classification of metabolites based on their broad functional roles, and panel (B) depicts the discrete classification of metabolites on their specific physiological role.

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enclosed spray chamber (DeVries Manufacturing, Hollandale, MN) calibrated to deliver 187 L ha^{-1} through an 8001E flat fan nozzle (TeeJet Spraying Systems Co., Wheaton, IL). As the GR_{50} of WAS and QUI biotypes were previously estimated as 151 and 59 g ae ha^{-1} respectively,[10] glyphosate was applied at a median rate of 80 g ae ha^{-1} (0.1× field rate). At the time of treatment application, the experimental units (plants) had similar morphological growth (height, leaf number, no vining). At 80 h after treatment (HAT), the three topmost young leaves, along with the apical meristem, from five replicates per treatment were destructively harvested and immediately frozen using dry ice blocks and stored at −80 °C until further analysis. To minimize the variations due to circadian rhythms, the plants of both the treatments were harvested 8 h after sunrise. The harvested tissues were ground to a fine powder with dry ice and stored at −80 °C.

Metabolite Profiling by GC-MS. Low-molecular weight polar metabolites in leaf tissues were identified and quantified using gas chromatography/mass-spectrometry (GC-MS) as described by Maroli et al. with slight modifications.[19] Briefly, metabolites were extracted

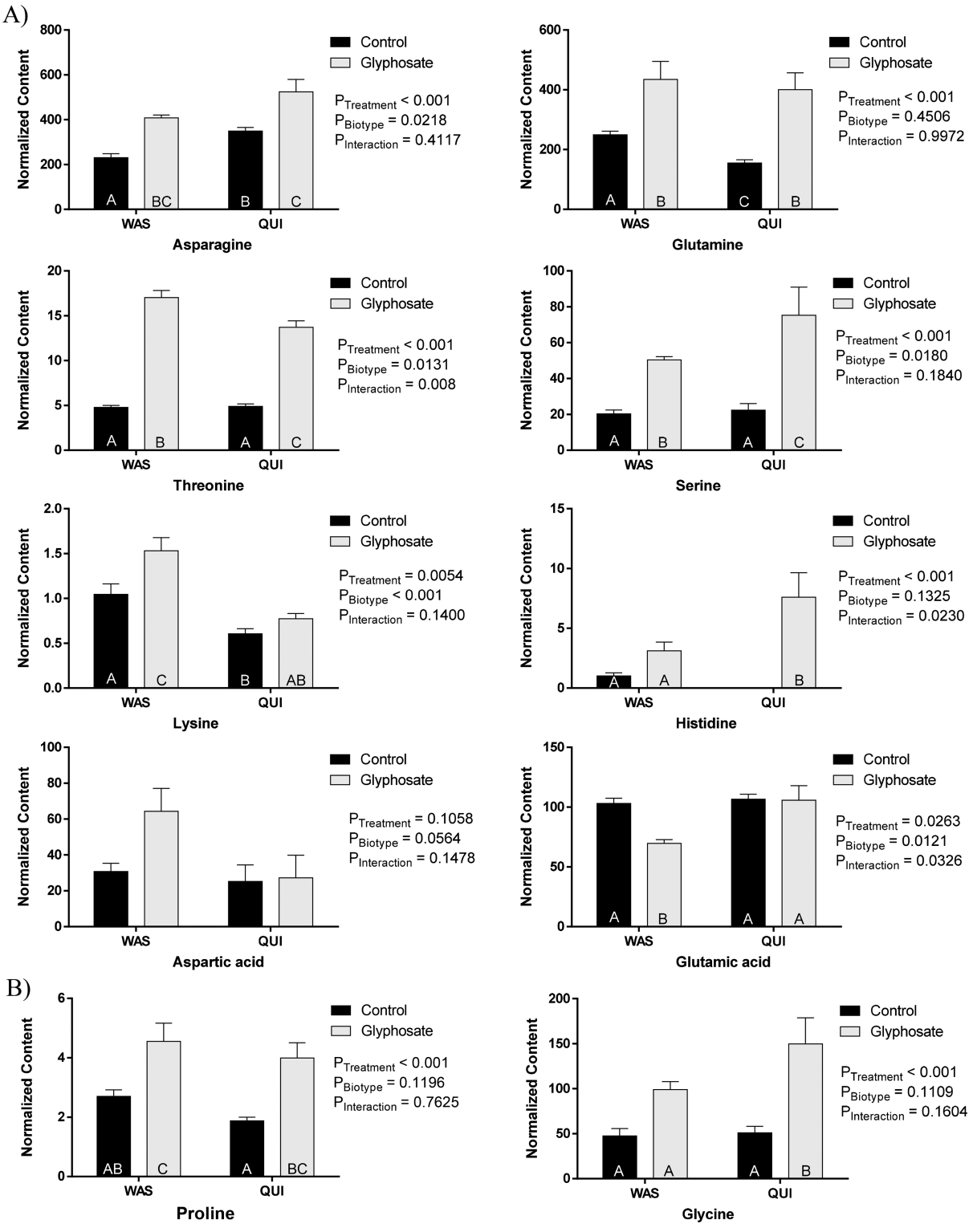

Figure 2. continued

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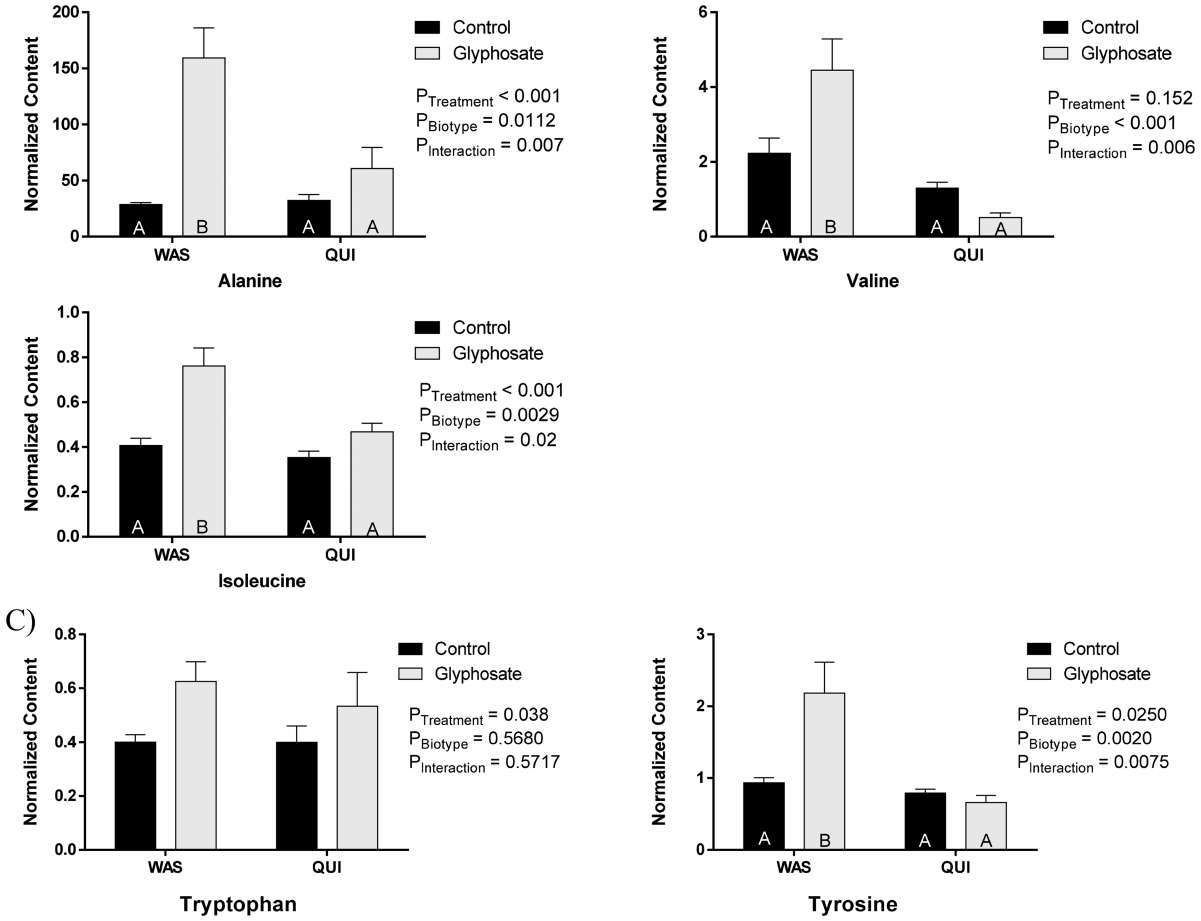

Figure 2. Influence of glyphosate on the amino acid and nitrogenous metabolites of water- and glyphosate-treated QUI and WAS biotypes of *Ipomoea lacunosa* leaves harvested at 80 HAT. Panel (A) represents comparative abundance of polar nonaromatic amino acids, while panels (B) and (C) represent comparative abundance of nonaromatic hydrophobic amino acids and aromatic amino acids, respectively. The data represents mean ± SEM of the normalized abundance and were tested by two-way ANOVA with Tukey's HSD comparing across all treatments and biotypes at a confidence level of 95%. Bars with the same letters are not significantly different at 95% confidence level.

with methanol from powdered leaf tissues by sonication. Following centrifugation and cooling, the resulting supernatant was transferred to prechilled glass tubes. Chlorophyll was removed by adding an equal volume of ice-cold chloroform. Polar metabolites were then partitioned into aqueous phase by adding half volume of cold water. A subsample of the top water−methanol phase was used for GC-MS analysis after chemical derivatization. The samples were spiked with ribitol (internal standard) and d27-myristic acid (retention time lock). The mixture was methoxylaminated with methoxylamine-HCl and then silylated with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and 1% trimethylchlorosilane (TMCS). The derivatized metabolites were analyzed using gas chromatography (Agilent 7980; Agilent Technologies, Santa Clara, CA) on a J&W DB-5 ms column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies), coupled to a transmission quadrupole mass detector (Agilent 5975 C series) with an electron ionization interface. GC-MS parameters are as follows: Initial oven temperature was maintained at 60 °C for 1 min, followed by temperature ramp at 10 °C per min to 300 °C, with a 7 min hold at 300 °C. Helium carrier gas flow was maintained at a constant pressure of 76.53 kPa and the injection port and the MS interphase were maintained at a constant temperature of 270 °C. MS quad temperature and MS source temperature were maintained at 150 and 260 °C respectively. The electron multiplier was operated at a constant gain of 10 (EMV = 1478 V), and the scanning range was set at 50−600 amu, achieving 2.66 scans sec^{-1}. An in-house metabolomics library supplemented with Fiehn Library (Agilent Technologies, Wilmington, DE, G1676AA) were used for peak identification while spectral deconvolution was performed using Automatic Mass Spectral Deconvolution and Identification System (AMDIS v2.71, NIST) using the criteria previously described by Maroli et al.[19]

Statistical Analysis. The changes in relative content of metabolites in response to the two treatments (biotypes and glyphosate) were analyzed using multivariate and univariate statistics. The ribitol normalized data were then processed using MetaboAnalyst.[20] Missing values were replaced with a value computed using the Bayesian PCA (BPCA) method.[21] Following data curation, the processed data were statistically normalized by averaging the values of each variable and dividing it by its standard deviation (auto scaling) to meet the assumptions of normality and equal variance.

Both supervised and unsupervised statistical analysis were performed to determine the statistical differences among the metabolites between the treatments. Unpaired *t* test with equal group variance was performed to identify potentially significant metabolites in discriminating the two treatments within the biotypes, while two-way ANOVA followed by Tukey's honest significant differences (HSD) posthoc test was employed to determine the statistical significance of the effect of biotype and glyphosate treatment on the individual metabolites. Differences among individual means were tested using Tukey's HSD multiple comparison tests with P_{value} < 0.05 considered statistically significant. Significance analysis of microarray/metabolites (SAM) was also performed to determine the metabolic differentiators across the treatments. For SAM analysis, the cutoff for significance, delta, was adjusted such that the false detection rate (FDR) was controlled at <5%. An unpaired fold change analysis was also calculated as the ratio between the glyphosate-treated group over the water (control) group. Any metabolite that is consistently above/below the specified FC threshold (threshold value of 2) is considered as significantly different (up/down-regulated). Two-way ANOVA and Tukey's posthoc analyses were performed using SigmaPlot for Windows v12.5 (Systat Software,

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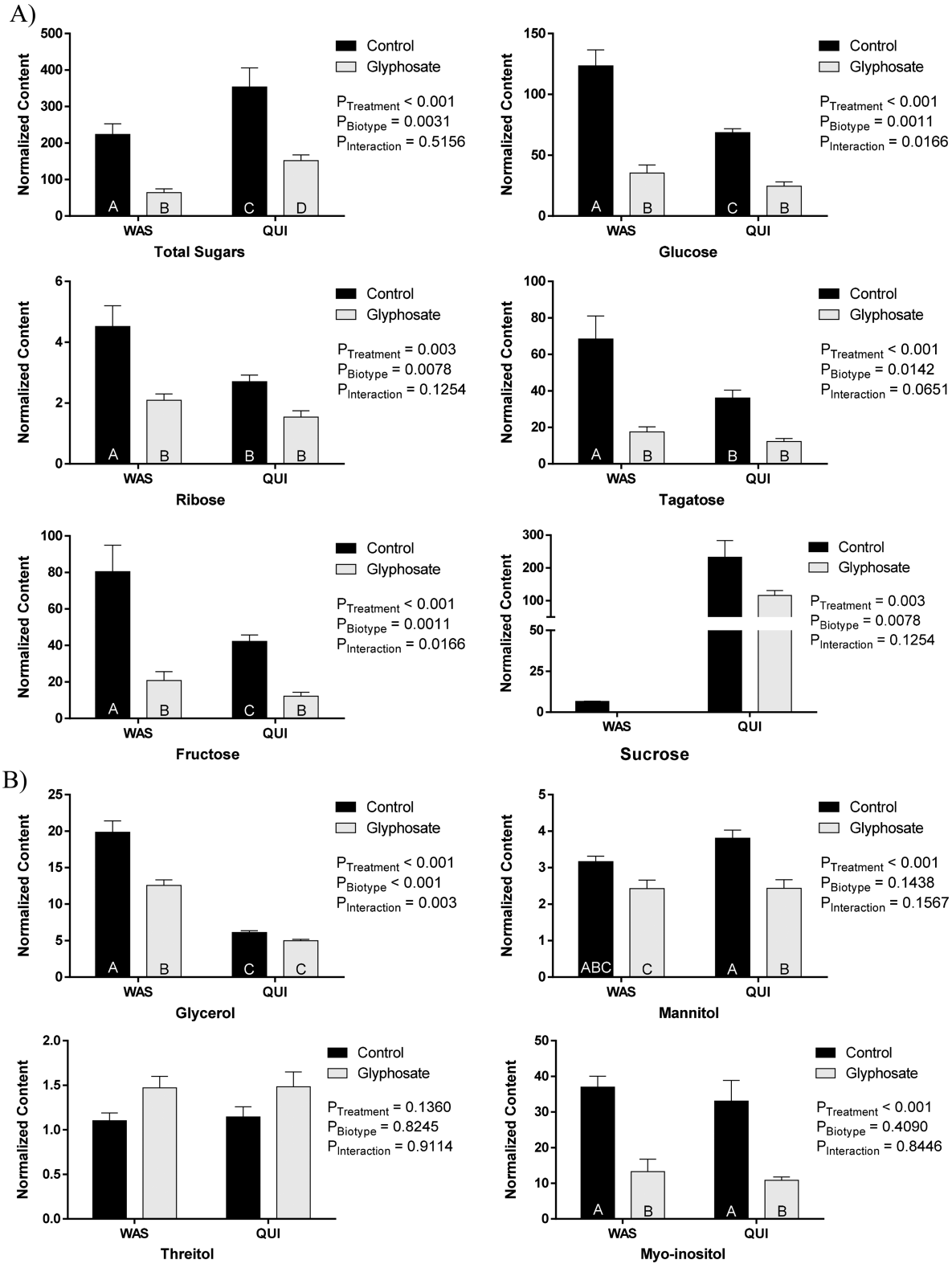
San Jose, CA), while *t* test, SAM, and fold change analysis were performed using MetaboAnalyst.[20] Graphs were constructed using GraphPad Prism v6.01 (GraphPad Software, La Jolla, CA).

For multivariate analyses, unsupervised principal component analysis (PCA) models were constructed to examine the significance of the effects of treatments on the metabolite profiles of the two biotypes. Hierarchical cluster analyses, using Euclidean distance as a similarity measure, were performed to determine the clustering of the metabolites in response to treatments. All multivariate analyses of the metabolite data sets were performed using MetaboAnalyst.

Pathway topological analysis comparing the differences in the metabolite abundance was performed using MetaboAnalyst, and the metabolic pathway maps were generated through KaPPA-View.[22] The global test algorithm was used for pathway enrichment analysis while the relative



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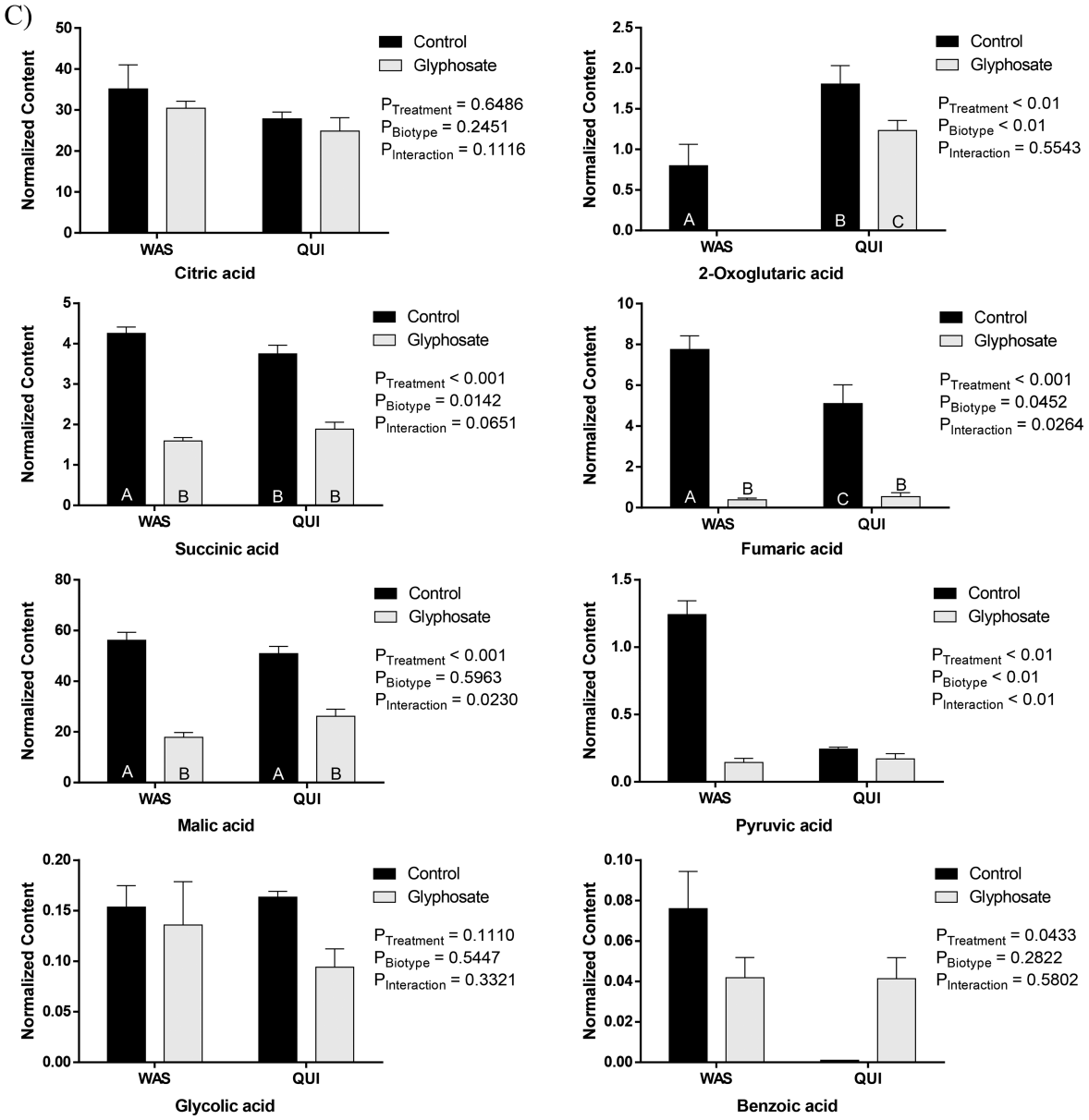

Figure 3. Influence of glyphosate on sugars, sugar alcohols, and TCA cycle metabolites in water- and glyphosate-treated QUI and WAS biotypes of *Ipomoea lacunosa* leaves harvested at 80 HAT. Panel (A) represents the total sugars content and the most abundant sugars, panel (B) represents the most abundant sugar alcohols (polyols), and panel (C) represents the TCA cycle metabolites and other organic acids identified in water and glyphosate treated WAS and QUI biotypes, respectively. The data represents mean ± SEM of the normalized abundance and were tested by two-way ANOVA with Tukey's HSD comparing across all treatments and biotypes at a confidence level of 95%. Bars with the same letters are not significantly different at 95% confidence level.

betweenness centrality algorithm was employed for pathway topological analysis. The *Arabidopsis thaliana* pathway library was used for pathway mapping. For pathway representation, the signal intensity of each metabolite was transformed to a log2 value and the ratio between the WAS biotype over the QUI-biotype were presented on the maps according to the documentation provided on their Web site (http://kpv.kazusa.or.jp/kpv4-kegg-1402).[22]

RESULTS AND DISCUSSION

Innate physiological differences between the two biotypes were determined by comparing the metabolic profiles of water-treated (control) WAS biotype with that of the QUI biotype. Ontological analysis classified the 56 metabolites detected by GC-MS into four broad functional groups (Figure 1A) of which 90% of the metabolites had a functional role in plant metabolism (Figure 1B). An unpaired *t* test computed with equal group variance at a P_{value} of 0.05 and a SAM[23] performed at a delta of 2 identified 11 significantly different metabolites between the two biotypes (Supporting Information Tables S1, S2). Pathway topological analysis further classified the 56 metabolites into 50 metabolic pathways (Supporting Information Table S3, Figure S1).

Innate Differences in the Composition of Nitrogenous Metabolites. Amino acids form the major constituent of nitrogenous metabolites in plants. Based on physiological concentrations, they could be broadly classified as major and minor amino acids.[24] Major amino acids such as Glu, Gln, Asp, Asn, Gly, Ser, Ala, and Thr are normally present in high concentrations, while amino acids such as Pro, His, Arg, Tyr, Trp, Met, Val, Phe, Ile, Cys, Leu, and Lys that are less abundant are considered minor amino acids. In the present study, Asn and Gln were the

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most abundant amino acids in both biotypes, but their relative content was inherently different between the two biotypes (Figure 2A). QUI had a higher content of Asn, while WAS had a higher content of Gln. As Asn and Gln are the primary nitrogen donors for amino acid biosynthesis, they are expected to be present in a higher amount in plants.[25] With respect to minor amino acids, compared to QUI, WAS had higher concentration of Pro, Lys, and His (Figure 2B). However, both the biotypes had similar abundance in aromatic amino acids (Figure 2C). The similarity in the aromatic amino acid profiles between the two biotypes in the absence of herbicide-stress is not surprising as similar trends were reported in studies comparing nonstressed glyphosate- susceptible and−resistant plants.[19,26,27] Other nitrogenous metabolites such as urea, citrulline, adenosine, uracil, allantoin, ornithine, and β-Alanine also did not inherently differ between the two biotypes (Supporting Information Figure S2).

Innate Differences in the Composition of Central Carbon Metabolites. The total content of the central carbon metabolites (sugars, sugar alcohols and organic acids) were similar across the two biotypes, although there were significant compositional differences within each group between the two biotypes. With respect to sugars, WAS had a higher abundant of ribose, glucose, fructose and tagatose, while the most abundant sugar in QUI was sucrose. Sucrose content of QUI was >10 times higher compared to that of WAS, and accounted to >75% of the total sugar content in QUI (Figure 3A). Sugar alcohols that are generally formed under reducing conditions from their analog sugars, did not differ between the two biotypes (Figure 3B). The two biotypes differed in their innate content of organic acids: α-ketoglutaric acid, fumaric acid, pyruvic acid, and benzoic acid (Figure 3C). While α-ketoglutaric (2-oxoglutaric acid) acid was ∼3 fold (95%) higher in QUI, fumaric acid and pyruvic acid was ∼1.5-fold (39%) and ∼3-fold higher in WAS (Figure 3C). Overall, as measured by the content of primary metabolites, the two Ipomoea biotypes with contrasting susceptibility to glyphosate exhibited differential cellular physiology in the absence of glyphosate stress. These differences were reflected at the compositional level within each metabolite class, than at the total content. While the aromatic amino acid content was similar across the two biotypes, the glyphosate tolerant WAS had a greater abundance of Gln and Pro. Compared to QUI, the compositional profile of central carbon metabolites were more diverse in the WAS, whereas sucrose, which is the main long-distance transport sugar in phloem, dominated the QUI profile. Benzoic acids are secondary metabolites that are derivatives of the shikimic acid pathway, although much is unknown about the pathways of benzoic acids.[28] Though the level of shikimic acid was similar between the two biotypes in the absence of glyphosate stress (Figure 4), the more tolerant WSA biotype had several-fold higher accumulation of benzoic acid (Figure 3C). However, under glyphosate stress, the level of benzoic acid increased in QUI whereas it decreased in the WAS, resulting in an overall similar content of benzoic acid in both biotypes after glyphosate treatment. The increase in content of hydroxybenzoic acids following glyphosate treatment has been reported in other species.[29]

Metabolic Perturbations under Glyphosate Stress. Supervised statistical analysis employing unpaired t test with equal group variance, fold change analysis and SAM analysis indicated 37 metabolites that are significantly different in the QUI biotype (Table 1) following glyphosate application while in the WAS biotype, about 33 metabolites were found to be affected (Table 2). PCA analysis showed that the metabolic profiles of the control and treated biotypes significantly changed as a result of glyphosate application (Figure 5A). The two-component model explained ∼61% of the total variance. The PC-1 axis differentiated between the two treatments (water and glyphosate), while the PC-2 axis delineated the two biotypes (QUI and WAS). The heatmap generated by unsupervised hierarchical clustering of potential candidates of importance extracted from the PCA model also showed significant changes due to glyphosate application (Figure 5B). Despite glyphosate tolerance, a 20-fold increase in shikimate accumulation was measured in both biotypes at 80 h after glyphosate application (Figure 4). This suggests that both biotypes had glyphosate sensitive EPSPS enzyme. This similarity in the buildup of shikimate also could suggest a potentially similar source-to-sink glyphosate translocation rates in both biotypes. This is consistent with the findings of Ribeiro et al., who also reported an increase in shikimic acid after glyphosate application and similar translocation rates between the two biotypes at 80 HAT.[10] Shikimate accumulation following glyphosate application have also been reported in glyphosate-resistant Palmer amaranth and horseweed.[19,30]

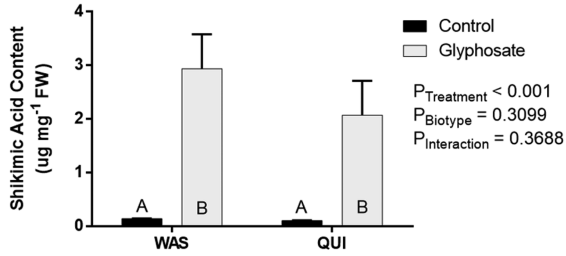

Figure 4. Shikimic acid content in water (control) and glyphosate treated WAS and QUI biotypes of *I. lacunosa* harvested at 80 HAT. The mean ± SEM was tested by two-way ANOVA with Tukey's HSD comparing across all treatments and biotypes at a confidence level of 95%. Bars with the same letters are not significantly different at 95% confidence level.

Changes in Amino Acid Metabolism under Glyphosate Stress. Physiological nitrogen assimilation initiates with either Gln or Asn and their respective acidic counterparts, Glu and Asp.[31] Following glyphosate application, compared to the respective water treated control, the concentration of Asn and Gln in WAS biotype increased by 77% and 129% respectively coupled with a 32% decrease in Glu (Figure 2A). Compared to the control, a similar increase of 81% and 158% in Asn and Gln, was similarly observed in glyphosate-treated QUI. However, no significant changes in Glu content was observed. The increase in Asn and Gln in response to glyphosate application in both the biotypes and a corresponding decrease in Glu in the WAS biotype could potentially be due to a lower utilization of these amino acids in downstream amino acid and protein synthesis, or due to proteolysis.[25] Compared to *Pseudomonas* strains that are less sensitive to glyphosate, a similar higher accumulation of Gln has been reported in glyphosate-sensitive strain of *Pseudomonas putida*.[17]

The available literature on the effect of glyphosate on free amino acid pools is conflicting, with several studies reporting increases in free amino acid pools following glyphosate application,[19,25,26,32,33] while other studies reporting a general decrease[34] or no change in the free amino acid profile.[35] The variations could be in part due to differences in plant species, glyphosate dose, and/or sampling time after treatment. In the present study, compared to the respective water-treated control, glyphosate application significantly increased the pool levels of polar amino acids such as Asn, Gln, Lys, Ser, and Thr in both the biotypes.

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Table 1. Statistical Analysis of Significantly Different Metabolites in QUI Biotype Following Glyphosate Application[a]

QG/QC	$t_{0.05}$	$P_{0.05}$	$-\log_{10}(p)$	FDR	$q_{0.05}$	$\log_2$(FC)
sugars						
allose	−3.0449	0.015945	1.7974	0.033293	0.008441	
fructose	7.2085	9.17×10^{-5}	4.0377	0.0016517	0.003724	−1.7919
glucose	9.5413	1.20×10^{05}	4.9194	0.00051474	0.003724	−1.7581
mannose	−2.7969	0.023308	1.6325	0.040407	0.008831	
ribose	5.8759	0.00037173	3.4298	0.0024306	0.00391	
sucrose	2.3654	0.045573	1.3413	0.072671	0.013789	−1.0901
tagatose	6.4377	0.00020092	3.697	0.0016934	0.003724	−1.9636
amino acids						
asparagine	−3.0055	0.016929	1.7714	0.033293	0.008441	
β-alanine	−2.7811	0.023885	1.6219	0.040407	0.008831	2.0253
glutamic acid	3.8795	0.0046778	2.33	0.013142	0.005054	
glutamine	−3.3939	0.0094448	2.0248	0.020639	0.006	
glycine	−3.4105	0.009216	2.0355	0.020639	0.005944	1.6394
histidine	−4.4833	0.0020468	2.6889	0.0092894	0.004841	11.052
isoleucine	−3.8163	0.0051169	2.291	0.013722	0.005163	
proline	−3.0099	0.016815	1.7743	0.033293	0.008441	
serine	−2.9271	0.019084	1.7193	0.03632	0.008649	1.5622
threonine	−9.0739	1.74×10^{-5}	4.7582	0.00051474	0.003724	1.2021
valine	4.221	0.0029117	2.5359	0.011199	0.004841	−1.1946
organic acids						
α-ketoglutaric acid	2.819	0.022527	1.6473	0.040407	0.008786	
benzoic acid	−3.9782	0.0040717	2.3902	0.013108	0.005054	3.5221
caffeic acid	4.6775	0.0015869	2.7994	0.0078025	0.00481	−5.762
dehydroascorbic acid	4.0376	0.0037484	2.4262	0.013009	0.004929	−1.065
fumaric acid	6.8512	0.00013082	3.8833	0.0016517	0.003724	−10.641
glyceric acid	4.3555	0.0024274	2.6149	0.01023	0.004841	−1.0637
glycolic acid	3.6289	0.006696	2.1742	0.015803	0.005288	
malic acid	6.7849	0.00013997	3.854	0.0016517	0.003724	
quinic acid	6.4451	0.00019935	3.7004	0.0016934	0.003724	−2.3462
shikimic acid	−2.1913	0.049797	1.0327	0.092842	0.017444	4.2276
succinic acid	5.7921	0.00040885	3.3884	0.0024306	0.00391	−2.838
sugar alcohols						
galactinol	3.9049	0.0045137	2.3455	0.013142	0.005054	
glycerol	2.6489	0.029306	1.533	0.04803	0.010396	
myo-inositol	4.1902	0.0030369	2.5176	0.011199	0.004888	−1.9134
threitol	−5.024	0.0010218	2.9906	0.0054804	0.004232	1.351
xylitol	5.7854	0.00041197	3.3851	0.0024306	0.00391	−8.0868
nitrogenous compounds						
allantoin	2.7788	0.02397	1.6203	0.040407	0.008831	
porphine	−3.7644	0.0055099	2.2589	0.014134	0.005181	1.2951
uracil	−3.9525	0.0042212	2.3746	0.013108	0.005054	4.8936

[a]Statistics were calculated as a ratio of the glyphosate treated (QG) over the control (QC) plants. An unpaired t-statistic ($t_{0.05}$) calculated at $P_{0.05}$ statistically discriminated between 37 metabolites. A false detection rate (FDR) was computed with a q-value (or p-value) of 0.05. The fold change (FC) is calculated on the $\log_2$ scale ($\log_2$(FC)), so that all changes (up/downregulated) will have the same distance to the zero baseline.

In addition to increase in Asn and Gln, the content of other polar amino acids such as Ser, Thr, and Lys also significantly increased. In WAS, compared to its control, the concentration of Lys, Ser and Thr increased by 46, 80 and 155% respectively. Similarly, in QUI, the content of Ser and Thr also increased by 150 and 80%, respectively (Figure 2A). Increase in Ser content after glyphosate application was also reported in glyphosate susceptible crop plants and agricultural weeds.[19,25,26,33,34] In these studies, the increase of Ser abundance was attributed to deregulation of C metabolism coupled with de novo N assimilation. Increase in Thr level is also significant because it is the main substrate for the synthesis of branched-chain amino acids in plants.[36]

Despite EPSPS inhibition, as evidenced by shikimic acid accumulation (Figure 4), there was no decrease in aromatic amino acid content in either of the Ipomoea biotypes upon exposure to glyphosate. While the Trp pool remained unchanged in both biotypes following glyphosate application, glyphosate treatment resulted in a 133% increase in Tyr concentration in WAS (Figure 2C). Increases in pools of aromatic amino acids following glyphosate application have been previously reported in other plant species,[16,19,25,37] and could be partly due to proteolysis or a lower downstream utilization, as supported by the increase in pool size of total free amino acids. However, unlike in plants, the aromatic amino acid content of soil bacteria is shown to consistently decrease upon exposure to glyphosate, and this herbicide toxicity could be reversed by the exogenous supply of aromatic amino acids in the growth medium.[17] Additionally, the study by Fernández-Escalada et al. showed that glyphosate treatment of glyphosate sensitive and resistant Palmer amaranth biotypes induced an accumulation of the transcripts corresponding

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Table 2. Statistical Analysis of Significantly Different Metabolites in WAS biotype Following Glyphosate Application[a]

WG/WC	$t_{0.05}$	$P_{0.05}$	$-\log_{10}(p)$	FDR	$q_{0.05}$	$\log_2$(FC)
sugars						
glucose	4.1787	0.003085	2.5107	0.00941	0.008035	−1.9263
fructose	3.9024	0.004529	2.344	0.012558	0.00854	−1.9505
ribose	3.439	0.008837	2.0537	0.020733	0.011126	−1.0211
sucrose	59.332	7.23×10^{-12}	11.141	4.41×10^{-10}	0.007142	−9.4888
tagatose	3.9272	0.004374	2.3591	0.012558	0.00854	−2.0096
amino acids						
alanine	−4.7482	0.001449	2.8391	0.005523	0.007366	2.5314
asparagine	−8.1771	3.73×10^{-05}	4.4284	0.000227	0.007142	
aspartic acid	−3.1038	0.014581	1.8362	0.029647	0.013928	1.6718
β-alanine	−4.6534	0.001638	2.7858	0.005876	0.007563	3.8282
glutamine	−3.7306	0.005783	2.2379	0.014697	0.008928	1.1489
glycine	−6.1204	0.000283	3.5481	0.001328	0.007142	1.3914
histidine	−4.2038	0.002981	2.5256	0.00941	0.008035	8.6507
isoleucine	−3.8672	0.00476	2.3224	0.012624	0.00854	
ornithine	−3.3811	0.009626	2.0166	0.021748	0.011375	
serine	−11.72	2.57×10^{-6}	5.5908	3.65×10^{-5}	0.007142	1.2635
threonine	−10.938	4.33×10^{-6}	5.3637	4.40×10^{-5}	0.007142	1.7181
tryptophan	−3.619	0.006793	2.168	0.016574	0.009142	1.1415
tyrosine	2.6324	0.030068	1.5219	0.053946	0.023423	1.1
valine	−2.9275	0.01907	1.7197	0.036351	0.017075	1.1877
organic acids						
α-ketoglutaric acid	3.0048	0.016946	1.7709	0.033346	0.016013	−6.4381
fumaric acid	10.652	5.29×10^{-6}	5.2768	4.61×10^{-5}	0.007142	−9.843
gluconic acid	3.3325	0.010346	1.9852	0.022539	0.011606	
glyceric acid	6.2928	0.000235	3.6297	0.001193	0.007142	−1.3723
malic acid	10.193	7.36×10^{-6}	5.1332	5.61×10^{-5}	0.007142	−1.6157
pyruvic acid	10.248	7.07×10^{-6}	5.1508	4.99×10^{-5}	0.007142	−3.1029
quinic acid	8.6283	2.52×10^{-5}	4.5979	0.000171	0.007142	−2.2786
shikimic acid	−2.5467	0.034349	1.4641	0.058203	0.024304	5.2385
succinic acid	13.948	6.76×10^{-7}	6.1698	1.38×10^{-5}	0.007142	−1.6076
sugar alcohols						
galactinol	5.941	0.000345	3.4616	0.001505	0.007142	
glycerol	3.207	0.01248	1.9038	0.026251	0.012684	
glycerol 1-phosphate	6.5522	0.000178	3.7494	0.000987	0.007142	−3.3171
myo-inositol	4.5287	0.001928	2.715	0.006532	0.007936	−1.4335
xylitol	22.251	1.76×10^{-8}	7.7546	5.37×10^{-7}	0.007142	−5.9042

[a]Statistics were calculated as a ratio of the glyphosate treated (WG) over the control (WC) plants. An unpaired t-statistic ($t_{0.05}$) calculated at $P_{0.05}$ statistically discriminated between 33 metabolites. A false detection rate (FDR) was computed with a q-value (or p-value) of 0.05. The fold change (FC) is calculated on the $\log_2$ scale ($\log_2$(FC)), so that all changes (up/down-regulated) will have the same distance to the zero baseline.

to genes of the aromatic amino acid pathway.[38] The study, based on enzyme and transcript analyses, further suggested a putative regulatory branch point in the pathway that could preferentially favor the flux of carbon toward Trp biosynthesis over Phe and Tyr biosynthesis.[38] However, consistent with our study, no accumulation of Trp was observed in the glyphosate treated palmer amaranth biotypes despite the preferential diversion of carbon toward Trp biosynthesis.

Accumulation of hydrophobic amino acids in response to abiotic stresses have been well documented in plants.[39] Glyphosate application increased the concentration of Ala, Val, Ile, and Pro in WAS, while only Pro and Gly increased in QUI (Figure 2B). Under glyphosate stress the Pro pool increased by 69% and 112% in the WAS and QUI biotypes, respectively. As the QUI biotype was exposed to a glyphosate dose higher than its GR_{50}, it would be expected to be under a greater stress. Hence, the higher proline accumulation in QUI, relative to WAS, could be potentially associated with stress mitigation adaptions. Of nonproteinogenic amino acids, only ornithine increased by 58 and 44% in WAS and QUI biotypes respectively (Supporting Information Figure S2). Consistent with our findings, the study by Moldes et al. also reported that in addition to Tyr accumulation, amino acids synthesized through shikimate-independent pathways such as Asn, Gln, Thr, Lys, Val, and Leu concurrently increased.[27]

Changes in Central Carbon Metabolites under Glyphosate Stress. Application of glyphosate increases the activity of 3-deoxy-D-arbinoheptulosonate-7-phosphate synthase (DAHP synthase), resulting in the uncontrolled carbon flow into the shikimate pathway, which in turn perturbs the central carbon metabolism in plants, and results in accumulation of metabolites upstream of EPSPS.[16] Resistance mechanisms involving translocation of glyphosate along the carbohydrate concentration gradient away from the target sites have been previously reported.[10,40−45] Primarily, glyphosate migrates along the phloem following sucrose movement.[44] In the present study, the glyphosate-treated QUI biotype had ∼50% decrease in total sugar content, whereas the WAS biotype had ∼75% decrease (Figure 4A). Glucose and tagatose decreased by more than 75% in both biotypes after glyphosate treatment. The innate higher abundance of sucrose

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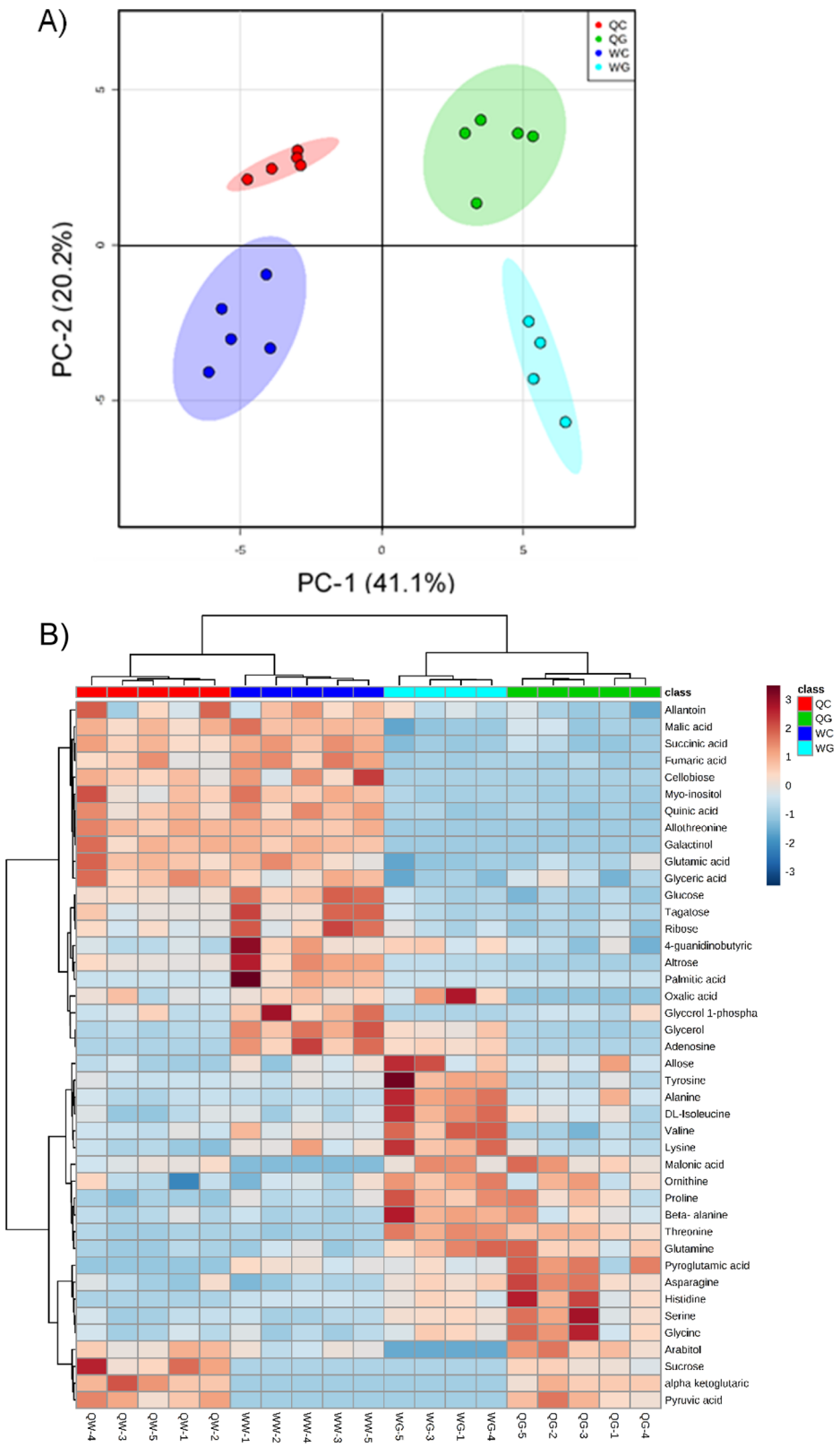

Figure 5. Differential grouping of water- and glyphosate-treated QUI and WAS biotypes of *Ipomoea lacunosa* based on metabolites identified at 80 HAT. Panel (A) represents the PCA score plot of metabolites in water- and glyphosate-treated QUI and WAS biotypes. The ellipses represent 95% confidence region. Panel (B) depicts the heatmap visualization of significant metabolite differences (ANOVA; $P_{value} < 0.05$) in WAS and QUI biotypes in response to water and glyphosate treatments following hierarchical cluster analysis. The algorithm for clustering was based on Euclidean distance measure for similarity and Ward linkage method for biotype clustering. ANOVA results comparing means of each metabolite across treatment groups, and false discovery rate (FDR) for multiple testing corrections are given in Supporting Information Table S3. WW, WG, QW, and QG correspond to WAS/water, WAS/glyphosate, QUI/water, and QUI/glyphosate, respectively.

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as the primary sugar source in QUI biotype could partly explain the increased translocation of glyphosate toward the target site as reported by Ribeiro et al. and consequently reduced glyphosate tolerance.[10] Accumulation of shikimic acid, consequent of EPSPS inhibition, is known to cause an unregulated flow of carbon into the shikimate pathway resulting in carbon substrate shortage for normal metabolism.[46] However, the observed decrease in sugars, concomitant with the increased pools of aromatic amino acids following glyphosate application (Figure 2C) could potentially delay the feedback regulation of upstream enzymes in the shikimate acid pathway such as DAHP synthase.[46] A similar physiological mechanism was reported to be basis of differential glyphosate tolerance of two biotypes of field bindweed to glyphosate.[47]

Of the primary organic acids, pyruvate, succinate, fumarate and malate, decreased in response to glyphosate application in both biotypes (Figure 3C). Compared to respective controls, succinate and malate decreased by ∼65% in WAS while in QUI, their levels decreased by ∼50%. In both the biotypes, fumarate decreased substantially following glyphosate application. In the glyphosate-treated WAS biotype, fumarate content decreased by 94% while in the similarly treated QUI biotype, fumarate content decreased by 89%. This decrease in fumarate could be attributed to its multiplicity of functions in response to stress such as serving as an alternative carbon sink for photosynthates, regulating pH for nitrate assimilation and acting as a respiratory substrate during senescence.[48]

Overall, the innate cellular physiology differentiated the Ipomoea biotypes with contrasting tolerance to glyphosate. The less tolerant QUI biotypes had a greater abundance of sucrose, the key carbon metabolites in phloem, which indicates a greater potential for source-to-sink transport of the herbicide in this biotype (Figure 6). The more tolerant WAS biotypes had a higher innate level of organic acids including pyruvate, and

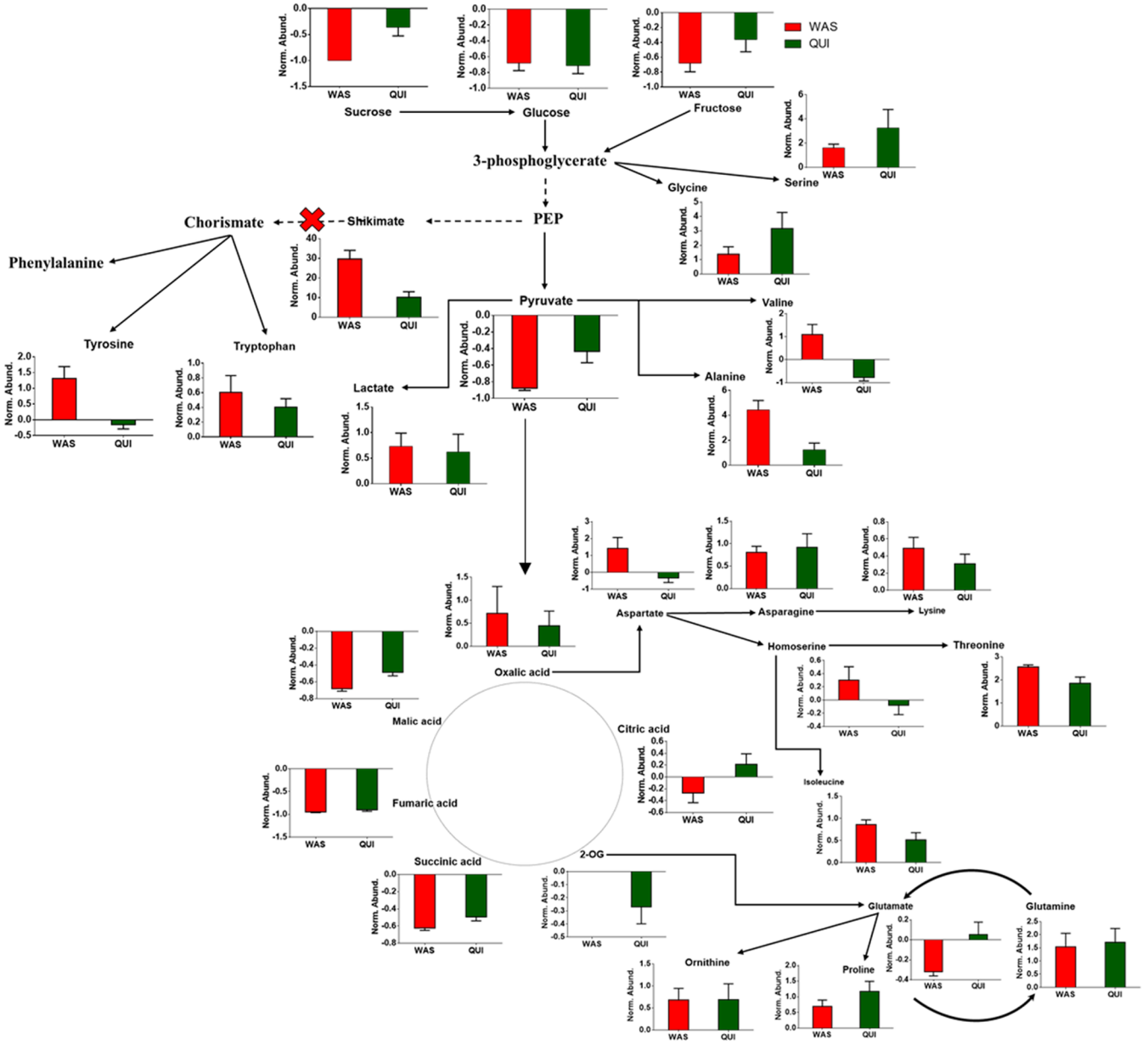

Figure 6. Metabolic pathway perturbations in glyphosate treated WAS and QUI biotypes of *I. lacunosa* harvested at 80 HAT. The red bars correspond to metabolites in WAS biotype, and the green bars correspond to metabolites in the QUI biotype. The data represents the mean ± SEM of the control normalized content.

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a more diverse sugar profile. However, despite the glyphosate tolerance, herbicide application induced similar and significant blockage of EPSPS enzyme in both the biotypes, resulting in metabolic perturbations in the carbon−nitrogen homeostasis. Thus, the present study illustrates the utilization of metabolomics to understand the differences in cellular physiology of weedy-biotypes that exhibit contrasting tolerance to glyphosate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b04722.

Significantly different metabolites between biotypes in control (water) treatment; pathway analysis of metabolites identified in control (water) treatment; univariate analysis of metabolites between water (control) and glyphosate treated biotypes; biochemical pathway representation of metabolites in control (water) treatment; normalized abundance of nonproteinogenic amino acids and nitrogenous metabolites (PDF)

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Notes

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■ ABBREVIATIONS USED

AMPA, aminomethylphosphonic acid; DAHP synthase, 3-deoxy-D-arbinoheptulosonate-7-phosphate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; FDR, false discovery rate; GC-MS, gas chromatography−mass spectrometry; HAT, hours after treatment; PCA, principal component analysis; SAM, significance analysis of metabolites/microarray; TCA, tricarboxylic acid cycle

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