Article

Direct Infusion Targeted Metabolomics of Phytochemicals in a Drought Tolerant Plant Introduction Soybean Cultivar

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ABSTRACT

Drought is the most prolific form of abiotic stress that legumes and cereal plants alike can endure; planting of an improper cultivar at the beginning of a season can cause unexpected losses up to fifty-percent under water deficient conditions. Herein, a plant introduction (PI) of an exotic cultivar of soybean (*Glycine max), PI 567731,* which demonstrates a slow wilting canopy phenotype in maturity group III was profiled in drought stress field trials in Missouri against a drought susceptible check cultivar, *Pana.* Relative phytochemical content of chlorophyll (chl) a/b, and pheophytin (pheo) was profiled by direct infusion electrospray Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry. High-throughput detection of metabolic profiles in twenty-four experimental groups occurred in triplicate within a few hours, without chromatographic separation. Subsequent multivariate analysis was able to form predictive models, encompassing the variance of growth and drought stress, within the experimental groupings at two physiological ages. Statistically significant increases within the Chl content in control conditions were detected, and an expanded photosynthetic antenna within the drought affected treatment condition could account for increased photosynthetic content; in particular, the distinct enhancement of chl b is noted from *PI 567731*. Moreover, the existence of unique chl-related metabolites (*m/z* >900) were confirmed through tandem mass spectrometry. The resultant coordination of fatty acids to the core of the porphyrin ring plays an unknown role in the proliferation of the photosynthesis, however the relative ratio of the most abundant chl-related metabolite is undisturbed by drought stress in *PI 567731,* in contrast to the drought susceptible cultivar.

KEYWORDS: plant introduction, drought stress response, slow wilting canopy, drought tolerance, chlorophyll content, phytochemicals, soybean, direct infusion, FT-ICR, metabolomics

ABBREVIATIONS

PI, plant introduction; SW, slow canopy wilting; MG, maturity group; QTL, quantitative trait loci; RIL, recombinant inbred line; Chl, chlorophyll; Pheo, pheophytin; GC/LC, gas or liquid chromatography; NMR, nuclear magnetic resonance; FT-ICR, Fourier transform ion cyclotron resonance; MS, mass spectrometry; DI,

direct infusion; ESI, electrospray ionization; CID, collision induced dissociation; 3D, three-dimensional; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; ODT, old drought treated; OC, old control; YDT, young drought treated; YC, young control

INTRODUCTION

Agricultural crops can endure a matrix of stress resultant from a variety of sources including biotic or abiotic stressors such as drought, flooding, salinity, or nutrient availability [1]. Among the different sources of stress plants can undergo, varying levels of water deficiency and drought have the most prolific and detrimental effect to agricultural farms on the global and national scale [2]. Numerous plant traits have been identified for the potential of improving the performance of drought-affected crops, mainly through conservation of water [3], with more recent works identifying the importance of slow canopy wilting (SW) phenotypes for their potential stress tolerance in water deficient environments [4]. Legumes, such as soybean (*Glycine max*), have a particular intolerance to water deficiency in the early stages of growth and flowering, where a decrease in water availability by half can result in up to a loss of half the expected yields [5]. Persistent changes in climate are predicted to have further detrimental impacts on agriculture in the coming decades [6].

Recently, an exotic soybean germplasm, *plant introduction* (*PI) 567731* in maturity group III (MG III), was identified to consistently express the SW phenotype in the field compared to the drought sensitive cultivar *Pana* [4,7]. *PI 567731* showed lower yield loss than *Pana* under drought stress with greater than 13% more yield index (yield under rain-fed/ yield under irrigation) [7]. *PI 567731* was found to use significantly less water under drought conditions, and this water conservation strategy was identified to be associated with limited-maximum transpiration rates. The transpiration of *PI 567731* was found to be sensitive to an aquaporin inhibitor (silver-nitrate) indicating the independence of a limited-maximum transpiration to a lack of silver-sensitive aquaporins in these SW genotypes. A major SW quantitative trait loci (QTL) (*qSW_Gm10*) was mapped on chromosome 10 from *PI 567731* through a genetic study in a recombinant inbred line (RIL) population and this QTL was further confirmed to delay canopy wilting under drought conditions in a near-isogenic background [4].

In efforts to understand many findings from field trials and further mapping of QTLs, many researchers have adopted use of proteomic and metabolomic techniques and platforms for data analysis to further reinforce and probe the mechanisms of plant stress responses. As resultant responses are characteristic to either acute or prolonged effects to drought stress, the initial impacts primarily effect net photosynthesis and photosynthetic performance of the plants. Under drought stress, stomatal closures and hormonal signaling through abscisic acid have been identified as the key reductants to net photosynthesis [8], with increased efficiency of the photosystem (PS) II denoted in stress tolerance [9]. Extended periods of drought stress have been demonstrated to also induce reduced Chlorophyll (Chl) content and related fluorescence

parameters [10], and are critical in considerations of the photosystem (PS) II, with drought stress also having noted to induce reordering the PSII core [11]. Even though water deficiencies do not directly impact the primary components of C3 plants PSI or PSII directly, these secondary impacts are well known in a variety of crops to reversibly impact photosynthesis, prior to photosynthetic decay [12]. This emphasizes the need for targeted approaches of profiling phytochemicals as a reliable means of screening for stress tolerances, including drought tolerance [13,14].

Both targeted and non-targeted approaches for determining metabolic profiles of soybean, and other agronomical crops have been entailed with instrumental approaches ranging from gas or liquid chromatography (GC/LC) coupled with mass spectrometry (MS) [15,16], nuclear magnetic resonance (NMR) [17,18], and a variety of spectroscopic techniques for in-vivo studies [9]. No one all-inclusive method for simultaneous detection of all metabolites is available. With a broad array of expression in a variety of primary and secondary metabolites in model plants and agricultural crops, methods either prove to be either moderate throughput with high specificity in extracts, or lack specificity with high-throughput analysis. High-resolution accurate mass MS platforms such as Fourier Transform ion cyclotron resonance (FT-ICR), due to unprecedented mass resolving power and mass accuracy [19], allows for the direct infusion of samples with no on-line separations [20]. In comparison to other MS profiling techniques, direct infusion FT-ICR holds at least a ten-fold decrease in analysis time, while simultaneously detecting hundreds of metabolic signals. As such, the platform is ideal for determination of relative phytochemical content. When utilized in tandem with physiological data and other interconnected pathways, insight on the acclimatization of photosynthesis in stress tolerances can be gleaned [21]. Herein described is the targeted profiling of phytochemical content and multivariate analysis of a drought tolerant cultivar, *PI 567731*, in comparison to a drought susceptible cultivar, *Pana,* grown in field trials, with drought treatment consisting of no irrigation or rainfall for three weeks.

MATERIALS AND METHODS

Materials

Quinapril HCl used was a USP Reference Standard (Rockville, MD). Methanol (HPLC Grade) was from Sigma-Aldrich (St. Louis, MO), and formic acid 88% (Certified ACS) was from Fisher Scientific (Fair Lawn, NJ) Whatman (Cat. 1001-055) filtration papers were used for vacuum filtration of particulate matter.

Samples Used During the Study

Two cultivars of soybean (*Glycine max)*, *PI 567731* and *Pana*, were grown in field trials at the University of Missouri (latitude 38.895305, longitude -92.205917). Two sets of the two soybean lines were grown in the field 20 meters away from each other under well-watered conditions. Sample collection was completed for

each of the two sets after 3 weeks without rain in the field. One set was irrigated 2 days prior to sample collection, which was considered as a control condition. The other set did not receive water either from rain or irrigation for 3 weeks, which was considered as drought condition. Leaf samples were collected at two physiological stages: young at V5 growth stage and old at R2 growth stage. After collection, leaves were flash frozen and transported at -80°C, and stored in polycarbonate petri-dishes at -20°C until extractions were processed.

Metabolite Extraction Protocol

The experimental and control groups underwent a respective pooling of plant tissue from the leaves collected. The samples were flash frozen with liquid nitrogen and macerated in an aliquot of solvent with an internal standard added. Maceration continued for five minutes and particulate matter was subsequently removed through vacuum filtration. Samples were dried in a vacuum oven at ambient temperatures and diluted to constant volume.

Data Collection and Processing

All spectra were acquired on a dual-source Bruker Daltonics 12T SolariX FT-ICR mass spectrometer (Bremen, Germany) by DI electrospray ionization (ESI) of the samples. Datasets for multivariate analysis were collected at 2 Mw using broadband detection from *m/z* 147.4 to 1500 with 100 scans for a transient of 0.8389 sec. Solvent extracts were analyzed in triplicate for instrumental and technical replicates of the two cultivars for both experimental conditions. Spectra were processed in DataAnalysis 5.0 with a signal-to-noise ratio of 5.0 for peak picking. Collision induced dissociation (CID) was used to confirm the identity of Chl-related metabolites, no charging additives were added due to sufficient signal in positive ionization mode. To reduce complex adduction, 0.1% of formic acid (v/v) was added during CID experiments at 4 Mw for a transient of 1.6778 sec with sufficient number of scans.

Statstical Analysis of Metabolites

The online web platform MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) was used for statistical analysis [22], and METLIN (https://metlin.scripps.edu/) was used for metabolite annotation [23]. Within MetaboAnalyst 5.0, a window of 2.5mDa was used to bin peaks from technical and instrumental replicates by DI FT-ICR MS. Peaks within samples with greater than 80% missing values were removed, and missing values were imputed utilizing an estimated limit of detection (one-fifth the average signal) for missing values. For multivariate analysis, filtering based upon standard deviation was completed, and normalization was completed to the peak area of the internal standard to remove variance from DI-ESI. Log fold changes were generated through transformation of the data.

RESULTS AND DISCUSSION

Multivariate Analysis of Cultivar Treatments

Principal component analysis (PCA) was performed as an orthogonal model to partial least squares discriminant analysis (PLS-DA) in order to discriminate subsets of drought treatment in the cultivars in MetaboAnalyst 5.0. Shown in Figure 1 are the three dimensional (3D) PCA of the entire sample population and models segmented according to the cultivar. This was completed to identify outliers initially from the 72 samples, with an average of 2,560 peaks per sample, after which further analysis was completed. The models demonstrate the distinct variance in the datasets from the control and drought treated metabolic fingerprints, as well as the unique variance to each cultivar, allowing for the distinction amongst the DI FT-ICR MS datasets.

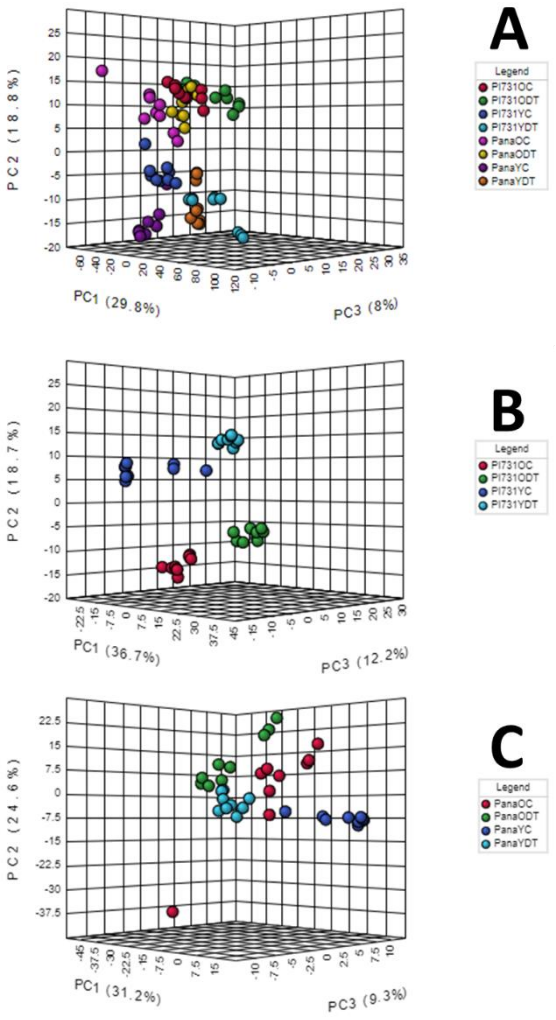

Figure 1. (A) is a 3D scores plot of the PCA of all *Pana* and *PI 567731,* (B) is the 3D-PCA of all *PI 567731* datasets, and (C) is the 3D-PCA of all *Pana* datasets. Each legend is unique to each scores plot, and is to the right of each plot.

Alongside of the drought treatment, the extra period of growth was also incorporated into the study to identify the influence of growth on the soybean plants. As shown in Figure 2A and 2B within PLS-DA models, the variance of growth was able to be separated. For the young

control (YC) and young drought treated (YDT) samples, the first component encompassed variance which separates the datasets, and the third component encompassed the variance imposed by drought itself based upon the groupings. These analyses utilize 95% confidence intervals in the scores plots, in the form of ellipses surrounding each subset population, allowing for the visual investigation of the analysis of variance between models. From the first component of the PLS-DA (Fig.2A, 2C), *Pana* datasets within the model had greater variance explained overall as well as both cultivars were distinguished by physiological age grouping. The third component of the model (Fig.2B, 2D), demonstrates overlapping confidence intervals in the case of *PI 567731*, with less observable variance within the metabolic fingerprint for both YDT/ODT, having compositions closer to that of the YC/OC in the third component. PLS-DA is the most commonly implemented tool in metabolomics datasets for a multitude of reasons; however, caution must be utilized with the raw data matrix, as the groupings can lend to a tendency to over fit the model when a variety of factors are not considered[24]. Based upon comparisons to the PCA, and other measures within MetaboAnalyst, this was not observed; as to not impart effects of growth on the further targeted analysis, OC/ODT were further analyzed independent of the YC/YDT datasets.

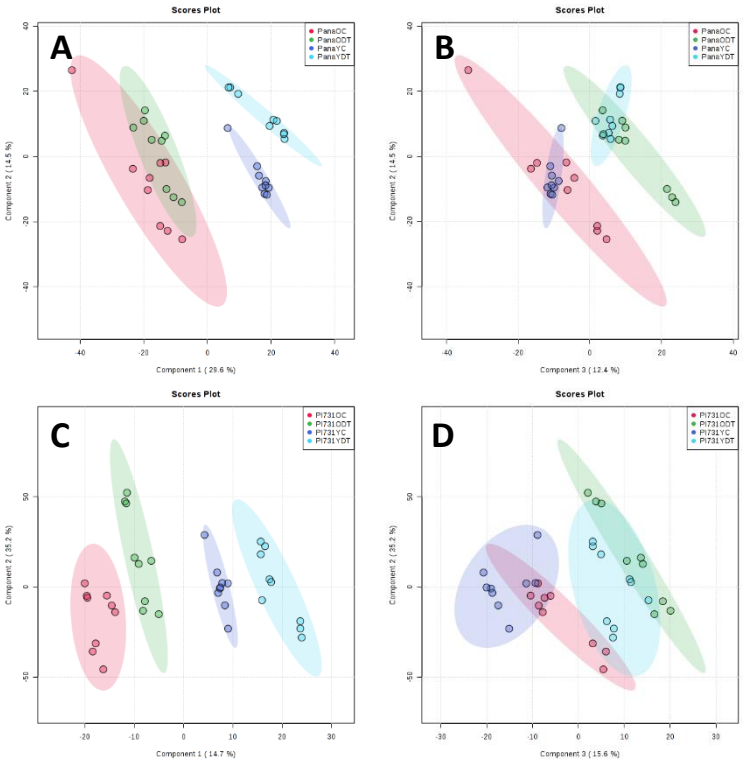

Figure 2. PLS-DA score plots of the first and second components (A, C) and the second and third components (B, D) of all subsets of *Pana* (A, B) and *PI 567731* (C, D) samples. With a window of 95% confidence around the encompassed variance.

Profiling Chlorophyll Content in the Treatments

PI 567731 was identified to preserve soil water through limited-maximum transpiration rates; this behavior would ultimately result in increased stomatal closures throughout the plant's life cycle, and decreased photosynthesis. Electron transport rates, carboxylation rates, respiration rates, and intrinsic water use efficiencies follow the dependencies of increased stomatal closures exhibited in water deficient events, with photosynthetic decay only occurring over extended periods of stress [25,26]. Throughout the profiling of the OC/ODT and YC/YDT datasets, it was observed that increased levels of Chl a/b, and Pheophytin (Pheo) a were

observed to be statistically enhanced components within the samples of *PI 567731*, with an overall median increase in relative abundance of Pheo a and Chl b detected within *PI 567731* as shown in the box and whisker plots in Figure 3.

T-tests were conducted for individual adducts for treatments of *Pana* and *PI 567731*. *PI 567731* OC was found to have statistically significant log fold increases ($p \leq 0.05$) for the sodiated adduct Chl b ($p = 0.010$) and Pheo a ($p = 0.0006$), as well as the protonated Pheo a ($p = 0.033$) in reference to *Pana* OC. Sodiated Chl b content was found to be significantly enhanced in *PI 567731* ODT ($p = 0.039$) in reference to *Pana* ODT. Distributions for the YC/YDT found significance for the protonated Chl a ($p = 0.0005$) in the YC of *PI 567731* and protonated form of Pheo a ($p = 0.019$) in the YDT of *PI 567731* over the corresponding *Pana* OC/ODT trials. These individual adducts account for the log fold distributions within the binned peak areas shown in the OC/ODT and YC/YDT plots in Figure 3, with most Pheo a levels remaining consistent, with highlights of elevated levels of Chl b for *PI 567731.*

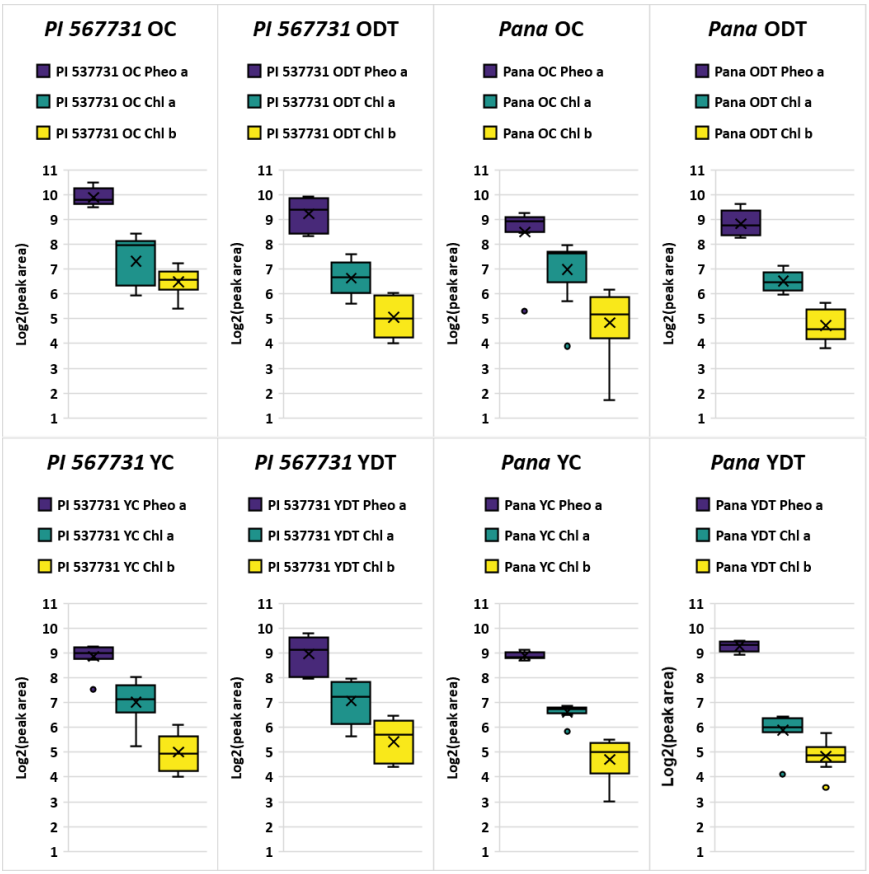

Figure 3. General log scale box and whisker plots of the binned protonated and sodiated adducts from *Pana* and *PI 567731* for Pheo a, and Chl a/b for OC, ODT, YC, and YDT. The solid black line represents the median, and black X represents the mean peak area for all subsets.

The average abundances and ratio of these metabolites has previously been demonstrated as secondary links to the holistic health of the plant, as demonstrated in previous works on Chl and related metabolites as markers for stress tolerance [27]. Increased total chlorophyll content was observed to be statistically significant by ANOVA ($p \leq 0.05$); previously this has been observed within positive chilling tolerance responses in maize [28]. With the noted increased yield index from the PI, alongside the QTL SW phenotypic expression, this increase in total chlorophyll content is indicative that chlorophyll content is up-regulated when the SW MGIII cultivar experiences drought stress, especially in earlier stages of growth. The observable enhancement was maintained under the drought treatment in younger populations of leaves, signifying that the three weeks of drought treatment with subsequent irrigation did not cause degradation, however it did cause a shift to the average Chl a/b ratios.

The enhanced expression of Chl b relative to *Pana* also marks proliferation of the photosystem, in agreement with increasing the breadth of the photosynthetic antennae in plants with positive stress responses [29]. The decrease within *PI 567731* for Chl b is also indicative of drought stress responses, while still remaining consistent with the drought susceptible control levels of *Pana* when *PI 567731* is under drought stress during later stages of growth. Within studies of maize and rice, with known water deficient intolerances, the photosynthetic antenna has been shown to be broadened, and an altered ratio of Chl a/b within tolerant cultivars is observed [30]. It has also been demonstrated Chl b is not just an accessory pigment in the light harvesting system, and can play a more pertinent role in primary light harvesting complexes [29], possibly allowing for more efficient use of light despite limited-maximum transpiration rate within earlier stages of growth. Moreover, the relative content should be at the same level of physiological responses from *Pana* if there was no existence of a positive stress response enhanced by a broadened photosynthetic antenna. The mapping of the SW QTL on chromosome 10 further suggests that in times of stomatal openings these increased levels of Chl content in the PI allow for effiecient photosynthesis.

Tandem Mass Spectrometry of Novel Chlorophyll Related

While further profiling the annotated metabolites, distinct novel Chl-related metabolites previously reported within soybean extracts were also denoted within this research, highlighted by Chl-related metabolite at *m/z* 1073.70740 [31]. As shown in the box and whisker plot in Figure 4, general log fold increases are experienced comparing young to older populations, with an increased median expression in drought treatment of the susceptible cultivar *Pana*. However, *PI 567731* levels of this sodium adduct remain relatively stable through drought stress response. Through the fragmentation of the molecules by collision induced dissociation (CID), these species (>893 Da) showed characteristic losses to that of a porphyrin ring base. Distinct moieties attached directly to the porphyrin ring are apparent by the neutral losses from the precursor ion, which still exhibits characteristic

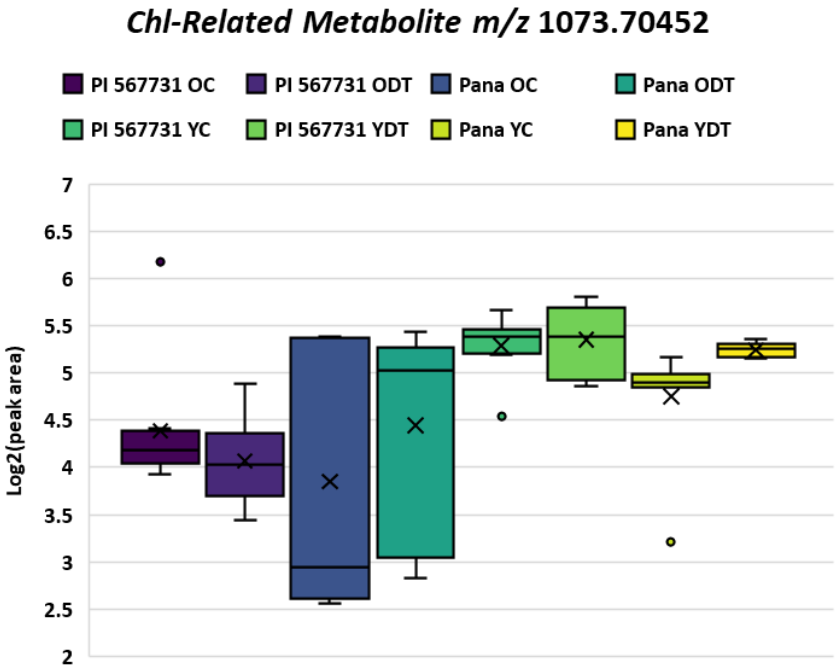

Figure 4. Box and whisker plot of Chl-related metabolite at *m/z* 1073.70483 relative log scale abundance in both *Pana* and *PI 567731* in both OC/ODT and YC/YDT datasets, legend above.

loss of the phytyl group ($C_{20}H_{38}$), as shown in the spectrum in Figure 5.

Once isolated, a product ion at 893.55473 Da forms, corresponding to a sodium adduct of Pheo a through the neutral loss of 180.15031 Da. When a neutral loss search within METLIN is completed with annotation in LIPID MAPS, this yields matches with a conjugated fatty acid ($C_{12}H_{20}O$). Upon further fragmentation, neutral losses of 278.29692 Da and 76.01606 Da appear, as annotated in Table 1, corresponding to the loss of the phytyl group ($C_{20}H_{38}$) to pheophorbide a, and further loss of moieties on the porphyrin ring ($C_2H_5O_3$), respectively. These results are consistent with previously reported literature studies of fragmentation of both Chl a and Pheo a by various dissociation techniques on FT-ICR MS [32,33]. A generation of a neutral loss under weak collisional energies (and the loss of an intact phytyl) suggests a weak coordination or bond formed to the porphyrin ring, not a modification that is not directly linked to the phytyl group. However, fractionation and NMR will be needed to confirm the fragmentation results.

Molecular Formula	Theoretical Mass	Observed Mass	Mass Error (ppm)
$C_{67}H_{94}N_4O_6Na$	1073.70656	1073.70483	1.61
$C_{55}H_{74}N_4O_5Na$	893.55514	893.55450	0.64
$C_{49}H_{64}N_4O_4Na$	795.48198	795.48019	2.21
$C_{35}H_{36}N_4O_5Na$	615.25779	615.25804	-0.40
$C_{33}H_{32}N_4O_2Na$	539.24175	539.24168	0.12

Table 1. Molecular formula, theoretical mass, observed mass, and calculated mass error in ppm for the precursor and fragment ions from the CID of chlorophyll related metabolite at *m/z* 1073.70483 with 30eV of collisional energy with argon as the collision gas.

Literature reports have noted increased fatty acid and lipid content within mesophyll membranes and chloroplasts to be essential for various stress tolerances [34], and are postulated to be pertinent to regulation of chloroplasts, especially under low or high temperatures, and abiotic stress [35,36]. This has a further influence upon Chl-protein complexes, with heterogeneous lipid and fatty acid composition of the membranes. With various Chl-related metabolites (>893 Da) being reported, fragmentation does confirm relation to Chl and Pheo in the porphyrin metabolism for these metabolites due to characteristic neutral losses; however, the metabolic pathway for the attachment and position on the porphyrin ring is unknown.

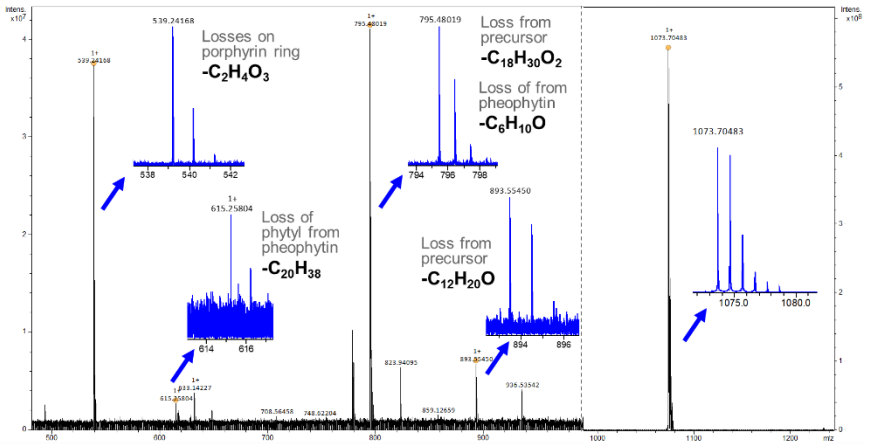

Figure 5. CID of chlorophyll related metabolite at *m/z* 1073.70483 at 30eV with argon collision gas, the precursor y-axis scale is x10^8 where fragment ions scale is x10^7. Characteristic losses of pheophytin occur with losses highlighted from either the sodiated pheophytin at *m/z* 893.55514 (0.64 ppm error) or the precursor chlorophyll related metabolite ion at *m/z* 1073.70483 (1.61 ppm error).

Considering that many of the species were also heavily oxidized, as apparent by high resolution accurate mass annotation of the full scan mass spectrum, this could also be a byproduct of reactive oxygen signaling (ROS) or of Chl-binding complexes in PSII membranes. Further study of these molecules is warranted.

CONCLUSIONS

Overall, *PI 567731*, a SW phenotype in MGIII with a profiled QTL on chromosome 10, was profiled in drought stress field trials against *Pana*, a drought susceptible metabolite. Multivariate analysis confirmed that the flux exhibited by drought stress detected by DI FT-ICR MS in the *PI 567731* was less extensive than that exhibited by the susceptible cultivar and more comparable to the control, forming predictive models for future analyses. Statistically significant increases within the Chl content in control conditions were also detected, and an expanded photosynthetic antenna within the drought affected treatment condition could account for increased photosynthetic content despite limited-maximum transpiration rates in the SW phenotype. With prior confirmation of the increased yield index and other physiological measures, profiling and observing the increased phytochemical content demonstrates the utility of this analysis in concert with physiological data for obtaining broad and focused profiled of metabolites. Furthermore, novel chlorophyll-related metabolites were probed within the analysis and confirmed with through tandem mass spectrometry to have fatty acids attached or coordinated as moieties to the porphyrin ring, with an unknown mechanism of attachment and relation into the porphyrin metabolism and photosynthesis.

DATA AVAILABILITY

The dataset of the study is available from the authors upon reasonable request.

AUTHOR CONTRIBUTIONS

KJZ, YH, HTN, and TDW conceived and designed experiments, YH raised and harvested plant specimens, KJZ performed the metabolomic assays using mass spectrometry, KJZ, YH, HTN, and TDW analyzed the data, KJZ wrote the manuscript, and KJZ, YH, HTN, and TDW revised and finalized the manuscript.

CONFLICTS OF INTEREST

The author(s) declare(s) that they have no conflicts of interest.

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