Evaluation of metabolite extraction protocols and determination of physiological response to drought stress via reporter metabolites in model plant *Brachypodium distachyon*

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Abstract: Metabolomics aims to systematically gather (quantitative) information on metabolites in the cell and is commonly viewed as the "missing link" between genomics, transcriptomics, and physiology. Typical metabolomics platforms consist of two main steps, the quenching and extraction of metabolites from plant material and the (un)targeted quantification of the extracted metabolites. *Brachypodium distachyon*, a native grass species of the Mediterranean region, is an attractive model plant to study temperate crops. In recent years, despite an increasing interest in genomic and transcriptomic studies, metabolomics studies for *B. distachyon* are still in their infancy. Drought, an abiotic stress factor, causes severe loss in plant productivity and it is therefore crucial to understand its effect on plant metabolism, in particular its metabolome. The objective of this study is to set up a quantitative plant metabolomics platform for *B. distachyon* to evaluate and optimize alternative metabolite extraction protocols (methanol and methanol-chloroform extraction). Focusing on three reporter metabolites (ATP, glucose, and starch) for metabolite quantification and two extraction protocols, we compared the drought stress response of 2 different genotypes from different geographical regions of Turkey. Results revealed that there is no "one-protocol-fits-all", pointing to significant differences in metabolite recoveries upon different extraction protocols, though methanol extraction generally yielded higher recoveries. Drought stress resulted in higher glucose levels while ATP and starch levels did not show statistically significant or consistent changes. In conclusion, the impact of drought stress on *B. distachyon* metabolism was significant and this study could be the basis for further metabolomics studies on plant stress response.

Key words: *Brachypodium distachyon*, metabolome analysis, plant metabolite extraction, drought stress, model plant

1. Introduction

Metabolomics links to physiology with the integration of DNA, RNA, protein, and metabolite analyses through metabolic pathways that are driven by enzymes and DNA encodes these enzymes. Thus, this integrative information generates an overall picture of the functional status of an organism (Dixon et al., 2006; Nikerel et al., 2012). The metabolome of an organism, in return, refers to the complete set of small-molecule chemicals and is a reflection of its metabolic state, hence giving information about the activated biological processes under particular conditions (Faijes et al., 2007). Metabolomics studies consist of 2 main stages: the extraction of metabolites from the cellular matrix and the quantification of the extracted metabolites. For successful metabolome studies, metabolite extraction is a key step in providing useful and reliable data. Significant efforts have been made for the selection of optimal metabolite extraction procedures for different platforms and organisms (Tambellini et al., 2013).

The world population is expected to exceed 9 billion by 2050 and global agricultural production needs to grow approximately by 70% in order to maintain the need for food supply (FAO, 2009). Environmental disasters, climate change, and other stress factors including cold, drought, and heavy metals or salinity, with limited land resources available for agricultural expansion, significantly worsen the current situation (Velthuizen et al., 2007). Improving plant adaptation to stress conditions is an urgent need to minimize yield loss of crops. Inducers creating stress in plants are divided into two major groups including abiotic stress factors (e.g., drought, salinity) and biotic stress factors such as pathogens. Drought, which can be described basically as water deficiency or incapability to access to water, is one of the greatest abiotic stress factors

rapidly increasing and threatening agriculture on a global scale (Ding et al., 2013). Plants activate a number of defense mechanisms to increase their drought tolerance in response to drought stress (Bartels and Sunkar, 2005; Ding et al., 2013). The complexity of stress tolerance limits the potential advances in this research area. Since model systems allow researchers to study complex processes, model plants have become valuable tools to study stress response.

The mechanisms of stress responses are quite complex; hence, understanding these mechanisms needs all the outputs of multi-omics approaches. In order to figure out the response mechanisms of plants to drought stress, several studies have been performed on transcriptomic and proteomic levels (Zheng et al., 2009; Budak et al., 2013). However, transcriptomic and proteomic approaches are insufficient in understanding the metabolic networks composed of biochemical reactions.

At the metabolomics level, many investigations have been carried out on abiotic stress response in plants. However, a great majority of these studies have been performed on *Arabidopsis thaliana*. Studies with *Arabidopsis* on temperature stress allowed significant progress in understanding temperature stress response mechanisms (Cook et al., 2004; Kaplan et al., 2004; Vannini et al., 2004; Gray and Heath, 2005; Morsy et al., 2007; Guy et al., 2008; Maruyama et al., 2009; Du et al.; 2010; Korn et al., 2010; Laura et al., 2010). In *Brachypodium distachyon*, stress response studies are largely focused on genomic and transcriptomic research, in particular with miRNAs (Wei et al., 2009; Zhang et al., 2009; Mochida et al. 2011; Tripathi et al., 2012; Jeong et al., 2013; Filiz et al., 2014; Ryu et al., 2014). However, there is a lack of information about drought stress response at the metabolomics level in *Brachypodium*. In order to provide a complementary approach and a bridge to physiology, it is required to get information on the metabolomics level.

Within the monocots, grasses that include cereal crops and forage grasses have major importance in the human diet, as animal feed, and for their capability of use as sustainable energy sources (Ozdemir et al., 2008; Brkljacic et al., 2011). *Arabidopsis thaliana*, as a dicot model plant, provided numerous scientific advances for almost 30 years; however, its applications are limited to monocot-specific research because monocots and dicots differ from each other in seed, root, and leaf structure and germination properties (Brkijacic et al., 2011). To date, the studies on *Brachypodium distachyon* have shown that as a model plant it can fill the research gaps on monocot species that *Arabidopsis* could not achieve. *Brachypodium distachyon*, as member of the grass family (Poaceae), has been proposed as a model plant for grasses since 2001 due to its well-suited model organism attributes, a compact genome,

5 pair of chromosomes, short generation time, easy genetic transformation, self-fertility, and simple growth requirements, together with its phylogenetic position, which makes *Brachypodium* attractive for improvement of temperate crops (Draper et al., 2001).

Brachypodium, as a wild-type cereal, has the potential to show wider variation for adaptation in terms of survival mechanism compared to domesticated cereals. This natural variation can be used potentially to uncover genes responsible for important traits in grasses. Schwartz et al. (2010) found substantial variation in flowering time among *Brachypodium* accessions. Drought tolerance is an important trait for sustainable crop production. Natural diversity in drought physiology has not been well characterized in most plant species. However, Luo et al. (2011) showed that 57 different accessions of *Brachypodium* represented different physiological responses to drought stress, such as chlorophyll fluorescence and leaf water content. They identified 4 groups of accessions differing in their sensitivities to drought stress.

In this study, we evaluated alternative extraction protocols and the effect of drought stress in model plant *Brachypodium distachyon* via reporter metabolites (specifically ATP, glucose, and overall starch) and focused on the quantification of these, rather than overall, high-coverage metabolome data.

2. Materials and methods

2.1. Plant material
The *Brachypodium distachyon* seeds were kindly provided by Prof Dr Metin Tuna of the Department of Field Crops, Namık Kemal University, Tekirdağ, Turkey. Two different genotypes of *Brachypodium* were used. Genotype 23 (labeled as G23) was sampled from the Şile/Ağva region at an elevation of 134 m with GPS location 41°05.347″N, 29°45.249′E. The second genotype, genotype 45 (labeled as G45), was sampled from the Kahta/Adıyaman region at an elevation of 202 m with GPS location 37°44′2.3″N, 38°32′0.2″E. Two populations of each genotype were used as plant material.

2.1.1. Plant growth conditions and drought treatment
Brachypodium plants were grown under a controlled environment (16/8 h light/dark photoperiod at 22/24 °C, relative humidity 60%–70%, and a photosynthetic photon flux of 320 µmol m^{-2} s^{-1} at canopy height provided by fluorescent lamps) in the greenhouse according to Filiz et al. (2009) until vegetative plants were developed. Drought stress was given to plants by withholding water for 7–10 days until yellowing of the leaves was observed morphologically while the control groups were watered every day until sample collection. Leaves of 5 individuals (5–10 leaves) in the vegetative state from one pot were pooled to be approximately 30 mg fresh weight of leaves.

Metabolite extraction protocols were applied to different plants. Each population was evaluated as a biological replicate to determine biological variance. Technical variance was evaluated with construction of a calibration curve and spiking experiments. In all cases, technical variation was found to be insignificant when compared to biological variation.

2.2. Plant metabolite extraction

In plant metabolomics studies, typically 2 metabolite extraction protocols are preferred among the others for the extraction of a broad range of metabolites, namely metabolite extraction using methanol (denoted as X-M) and metabolite extraction using methanol/chloroform (denoted X-MC). Extraction efficiencies of these two methods were evaluated.

2.2.1. Metabolite extraction using methanol

Polar metabolites were extracted from homogenized samples according to the protocol of Roessner and Dias (2013). Briefly, samples were incubated in 100% methanol solution at 70 °C for 15 min and centrifuged at 14,000 rpm for 15 min. Supernatant was separated (named sp1), the pellet was rewashed with double distilled water and centrifuged again at 14,000 rpm for 15 min, and the supernatant was combined with sp1 and labeled as sp12. Two additional recovery steps with water were implemented in addition to the original procedure, and each time the supernatants were combined to check whether metabolites could further be recovered with additional washing steps. Combined supernatants collected from each step were labeled as sp1, sp12, sp123, and sp1234, referring to the number of washing steps.

For the ATP assay, aliquots of 30 µL were taken from each sample and the remaining supernatants were dried under a speed vacuum concentrator. Dried samples were used for glucose analysis in high-performance liquid chromatography (HPLC, Agilent, USA). All samples were stored at –80 °C until used.

2.2.2. Metabolite extraction using methanol/chloroform

Metabolite extraction from homogenized samples was performed according to Gomez et al. (2002). Cold extraction solvent consisting of chloroform:methanol:water (1:2.5:1 v/v/v) was added to each homogenized sample. Samples were shaken (5 min, 4 °C) and centrifuged (14,000 rpm, 2 min), and supernatants were collected (upper phase 1). Distilled water was added, vortexed, and centrifuged (14,000 rpm, 2 min) and upper phases were collected. For ATP assay, aliquots of 30 µL were taken from each sample and the remaining supernatants of methanol:water phases were dried under a speed vacuum concentrator and stored at –80 °C until used for glucose analysis in HPLC. Lower phases containing chloroform were prepared for the determination of starch level (Gomez et al., 2002).

2.3. Analytical methods

2.3.1. Determination of ATP level

ATP was used as a reporter metabolite to evaluate the efficiency of metabolite extraction protocols, the effect of drought stress, and the responses of different genotypes. ATP in each sample was quantitatively measured using a commercial kit (Promega ATP Assay Kit) and a luminometer (Luminoskan Ascent Thermo), according to the manufacturer's instructions. After preparing a calibration line with known ATP standards vs. relative light unit (RLU) values, the RLU for the samples with unknown ATP levels were measured and the calibration line was used to obtain concentration estimates. To rule out matrix effect, increasing concentrations of ATP standard were added to individual aliquots of the selected samples.

2.3.2. Determination of starch content

Starch was used as another reporter metabolite to evaluate the effect of drought stress and response of different genotypes. Lower phases of the samples containing chloroform, which were extracted by methanol:chloroform extraction, were briefly washed with methanol and centrifuged according to the procedure of Gomez et al. (2002). Samples were dissolved in 100 µL water to determine starch content. The starch calibration curve was prepared with known starch concentrations (0, 0.75, 1.5, 3 g/L) and after mixing with 1.5 mL of iodine solution absorbances were read at 550 nm by spectrophotometer. Since the starch contents of the samples stayed below the limit of detection of the assay, increasing concentrations of starch solution (3, 1.5, 0.75 g/L) were spiked to individual aliquots (20 µL) of each sample to achieve detectable levels and the absorbances were read. The resulting data (added starch as x and absorbance as y) were used to construct a line (details are given in Section 2.4) for each sample. Starch concentration in the sample was determined from the y-intercept of this line.

2.3.3. HPLC analysis for determination of glucose content

Previously dried samples content were dissolved in 200 µL of acetonitrile:HPLC water mixture (1:1 v/v) for HPLC analysis. Increasing concentrations of glucose standard (5, 7.5, 10 g/L) were added to individual aliquots of each sample. HPLC analysis was performed with the Agilent 1100 HPLC system with refractive index detector using the Zorbax Carbohydrate Analysis Column, 4.6 × 250 mm (Agilent, USA). Conditions were set as 1400 mL/min flow rate, 30 °C, eluent 75 (acetonitrile):25 (HPLC water). Glucose levels were estimated via a constructed log-linear model using measured peak area from HPLC measurements from the intercepts of the calibration curves.

2.4. Statistical analysis

2.4.1. Determination of concentration of reporter metabolites from spiking experiments

For all three reporter metabolites, spiking experiments were performed as described above, in order to rule out matrix effect and provide an estimate for the metabolite level for concentrations below the detection limit. Resulting data as metabolite added (x) vs. measurement (y) were used to fit a line y = β_0 + β_1, using simple linear regression. The estimates for the coefficients were calculated using the following equation.

$$\widehat{\beta_1} = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sum_{i=1}^{n}(x_i - \bar{x})^2}, \quad \widehat{\beta_0} = \bar{y} - \widehat{\beta_1}\bar{x} \quad \text{with}$$

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i \quad \text{and} \quad \bar{y} = \frac{1}{n}\sum_{i=1}^{n} y_i$$

A concentration estimate for the reporter metabolite was given by the y-intercept, β_0, as the measurement without any standard addition (x=0). This linear model was first tested for significance under the null hypothesis that both parameters were zero. The confidence interval for they -intercept was calculated as:

$$\widehat{\beta_1} = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sum_{i=1}^{n}(x_i - \bar{x})^2}, \quad \widehat{\beta_0} = \bar{y} - \widehat{\beta_1}\bar{x} \quad \text{with}$$

$$s_{\widehat{\beta_0}} = \sqrt{\frac{1}{n(n-2)}\left(\sum_{j=1}^{n} \hat{\varepsilon}_j^2\right) \cdot \frac{\sum_{i}^{n} x_i^2}{\sum_{i}^{n}(x_i - \bar{x})^2}}$$

where $\hat{\varepsilon}_j$=y_i-(β_0+$\beta_1 x_i$) and n is the number of spiked samples.

2.4.2. Determination of significant change in reporter metabolite levels

After estimating metabolite levels from individual samples, statistically significant changes in metabolite levels upon extraction protocols or drought treatment were determined. For this, a simple t-test was used with the null hypothesis that there was no difference in metabolite levels upon extraction method or drought treatment. The statistical significance of the change was assessed by the calculated t-statistic as:

$$t = \frac{x_i - x_j}{s_{ij}} \quad \text{with} \quad s_{ij} = \sqrt{\frac{s_i^2}{n_i} + \frac{s_j^2}{n_j}}.$$

Here, the subscripts i and j refer to different extraction protocols or drought treatment, x_i and x_j represent the

reporter metabolite concentration, and s_i and s_j represent the estimate of standard deviation in the corresponding measurement of reporter metabolite over the different samples of a genotype. The resulting t-statistic was compared to the critical α level, taken as 0.1 (90% confidence). The degree of freedom for the t-statistic was provided by Welsch approximation as:

$$df = \frac{\left(s_i^2/n_i + s_j^2/n_j\right)^2}{\left(s_i^2/n_i\right)^2/(n_i-1) + \left(s_j^2/n_j\right)^2/(n_j-1)}$$

3. Results

3.1. Evaluation of technical variation, matrix effect, and optimization of methanol extraction procedure

The technical variations due to instruments were evaluated by repeated measurement of the same sample and by inspecting the goodness-of-fit of the constructed calibration line for each metabolite. The matrix effect was evaluated by spiking experiments. The presence or absence of matrix effect was assessed by comparing the y-intercept of the constructed line with the actual measurement from spiking experiments without any spiking, in the case that such data were available. The results of these analyses are presented in Figures 1A–1C, where typical data from a spiking experiment for ATP (Figure 1A), starch (Figure 1B), and glucose (Figure 1C) are presented. In the case of starch and glucose, the metabolite level without any spiking was found to be below the limit; therefore, the data from spiking experiments were used to provide an estimate for the metabolite level (presented as black squares on y-axes in Figure 1). Since the levels of both glucose and starch were low, the metabolite levels were assumed to follow a log-normal distribution, allowing a theoretical minimum of zero for both quantities.

The methanol extraction (X-M) procedure originally consisted of 2 steps including methanol and water treatment (described in Section 2.2.1). After removing all insoluble molecules in pellets, supernatants were combined for large metabolome recovery. Here, ATP levels from supernatants collected in each recovery step were measured to understand whether the addition of these steps led to the obtaining of higher amounts of metabolites (Figure 1D).

The same modified extraction procedure was carried out for 2 different *Brachypodium* genotypes (Figure 1D, G23 and G45, solid and dashed lines, respectively). 'sp1' refers to the first supernatant collected after methanol treatment, while sp12, sp123, and sp1234 refer to the combined supernatants with subsequent recovery steps using water. A scarce amount of ATP was obtained from sp1, followed by significant increase in ATP level in sp12. Additional steps (sp123 and sp1234) yielded approximately 5%–15%
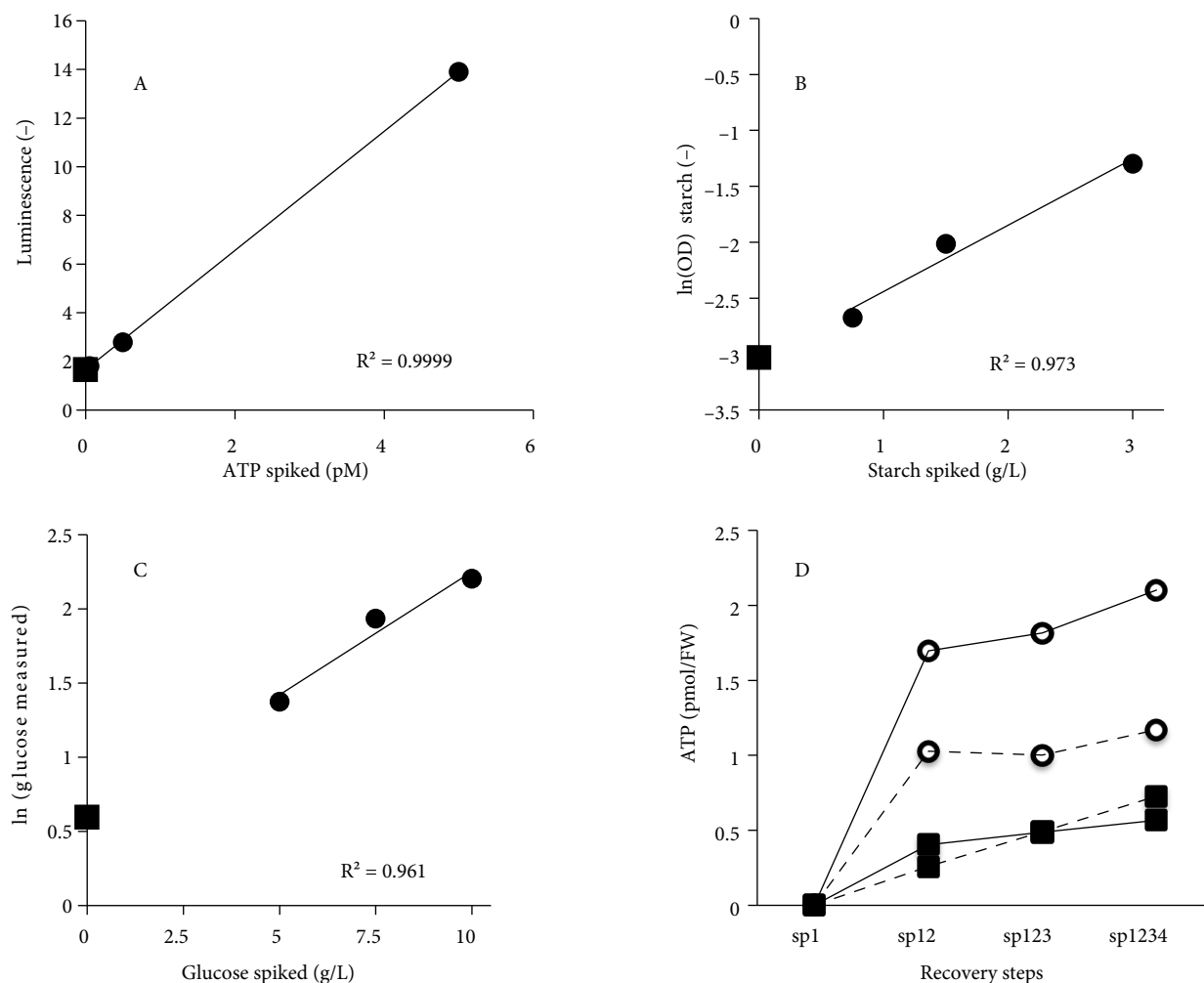
Figure 1. (A) Typical results of the ATP spiking experiment. The point estimate of the actual concentration was calculated using the square on y-axis. Data were taken from one population of G23 (normal conditions, X-M). (B) Results of the starch spiking experiment. Since the starch levels were below the detection limit, the constructed line was used to estimate the starch level (marked with a black square on the y-axis). Data were obtained from one population of G23 (normal conditions, X-MC). (C) Results of the glucose spiking experiment. Data were obtained from one population of G23 (normal conditions, X-M). (D) The effect of additional recovery steps in the X-M method on ATP yield for the two genotypes (solid lines represent G23 and dashed lines represent G45) under two treatments (open circles represent normal conditions, filled squares represent drought-treated samples).

increase in ATP levels; similar trends were observed for both genotypes under normal and drought conditions. Based on these results, technical variation was observed to be negligible compared to the biological variation, matrix effect was minimal, and three recovery steps (up to sp123) were further accepted as a (minor) protocol modification.

3.2. Comparison of metabolite extraction protocols

The two metabolite extraction protocols (X-M and X-MC) were compared via the statistically significant change in reporter metabolite levels for both genotypes. For ATP, the comparison of the metabolite levels is presented in Figure 2. The following P-values were found under the null hypothesis that the extraction method had no effect and

the alternative hypothesis was that X-M protocol yielded higher levels of ATP: 0.14 and 0.03 for G23 under normal and drought conditions, respectively, and 0.07 and 0.07 for G45 under normal and drought conditions, respectively. Overall, X-M yielded higher ATP levels.

Glucose concentrations, estimated via a constructed log-linear model (typical plot is presented in Figure 1C), between 2 different conditions (drought and normal) and 2 protocols are presented in Figure 3. Similar to the previous case, the statistical significance was assessed via the P-value under the null hypothesis that the extraction protocol yielded the same glucose levels, and the alternative was that X-M yielded higher glucose level
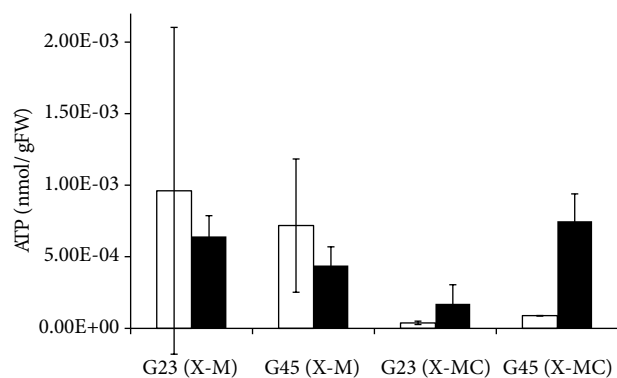
Figure 2. Comparison of measured ATP levels for both G23 and G45 obtained at the third recovery step (sp123) with different extraction protocols (X-M and X-MC) under normal (white bars) and drought (black bars) conditions. The error bars represent the standard deviation over the populations of the corresponding genotype.

Figure 3. Comparison of estimated glucose levels for both G23 and G45 using different extraction protocols (X-M and X-MC) under normal (white bars) and drought (black bars) conditions. The error bars represent the standard deviation over the populations of the corresponding genotype.

than X-MC. The following P-values were obtained: 0.0006 and 0.0198 for G23 under normal and drought conditions, respectively, and 0.18 and 0.08 for G45 under normal and drought conditions, respectively. Overall, X-M yielded higher glucose levels.

3.3. The effect of drought treatment on reporter metabolites

The effect of drought treatment on ATP, glucose, and starch was also assessed by determining statistically significant changes in metabolite levels. Focusing on ATP levels, drought stress did not yield significant changes in its level (Figure 2). The P-values were found to be 0.276 and 0.106 for G23 for X-M and X-MC treatments, respectively, and 0.191 and 0.013 for G45 for X-M and X-MC treatments, respectively. As for glucose, the drought stress resulted in a significant increase in its levels: the P-values were found to be 0.041 and 0.022 for G23 upon X-M and X-MC protocols, respectively, and 0.300 and 0.051 for G45 upon X-M and X-MC protocols, respectively (Figure 3). Lastly, despite the slight increase in starch levels, drought stress resulted in no statistical change in starch levels when considering the variations within different members of each phenotype (P-values were found to be 0.300 and 0.283 for the G23 and G45 genotypes with the X-MC protocol; Figure 4).

3.4. Comparison of metabolomics response of two genotypes to drought stress

Taking Figures 2–4 together, both genotypes responded similarly to drought stress: both glucose and ATP levels increased (Figures 2 and 3, G23 (X-MC) and G45 (X-MC) columns), while no significant or systematic change in starch levels was observed (Figure 4), when the X-MC protocol was used. However, this pattern is in
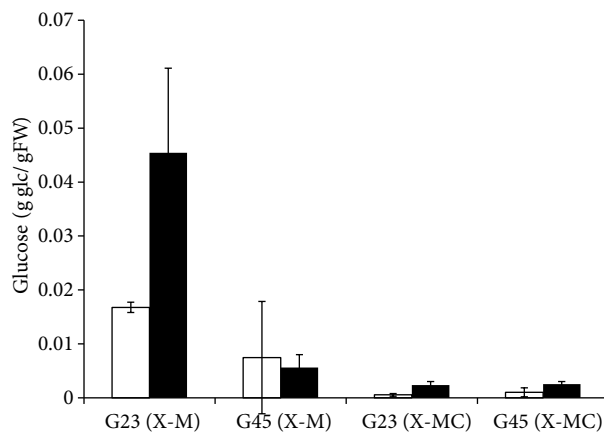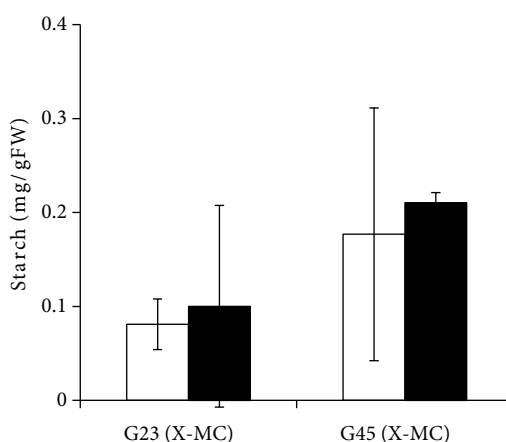

Figure 4. Comparison of estimated starch levels between untreated (white bars) and drought-treated (black bars) samples via the X-MC protocol. The error bars represent the standard deviation over the populations of the corresponding genotype.

contradiction for ATP (drought stress results in decrease in ATP) when the X-M protocol is considered. Generally, the G45 genotype yielded lower ATP levels (Figures 1D and 2, X-M treatment) than G23. The glucose levels from G23 and G45 were not statistically different: the corresponding P-value was found to be 0.123 and 0.202 for X-M and X-MC protocols, respectively, under normal conditions, and 0.023 and 0.290 for X-M and X-MC protocols, respectively, under drought conditions. Similarly, starch levels were found to be similar (P-values of 0.161 and 0.103 for normal and drought conditions, X-MC protocol, respectively).

4. Discussion

In biological analyses, components of matrixes in biological samples might influence the response of analytes of interest and lead to inaccurate quantitation, known as the matrix effect (Chiu et al., 2010). Specifically for ATP levels, even if the proteins and other particles are effectively removed from the sample, still many substances such as phospholipids remain, releasing phosphate molecules and possibly causing matrix effects. Standard additions of analytes of interest (in this case ATP) into each sample can be used to take the matrix effects into account (Shariati-Rad et al., 2013). In the samples analyzed in this study, inspecting the results of spiking experiments, the matrix effect was absent. Results of standard addition experiments confirmed that measurement of ATP levels within the plant matrix was reliable and highly accurate.

Providing an estimate for metabolites with very low levels (below the detection limit) is a well-studied challenge. These studies typically yield censored distributions, as the values are typically not reported (Succop et al., 2004). In this work, for metabolites near or below the detection limit, log-normal distribution was assumed, allowing a theoretical minimum of zero. As such, for samples with low concentration, a log-linear line ($y=\beta_0+\beta_1\,z$ with $z=\ln(x)$) was constructed for each sample using data with increasing concentrations of metabolite standard added to individual aliquots (Supplementary Figure 1 for ATP; Nischwitz et al., 2003). This allowed providing an estimate for metabolites that was below the detection limit. The advantage of this approach is that it also results in a confidence interval, though sometimes broad, for the measurement estimates.

High extraction efficiency is an obvious must for high-quality plant metabolome studies. The efficiency would depend on solubilities of metabolites of interest, the cell wall structure, which in turn changes upon geographical adaptation of plants. Indeed, Luo et al. (2011) found significant phenotypic variation in response to drought stress between different *Brachypodium* accessions. In our experiments, the 2 genotypes were sampled from different geographical regions and climates of Turkey (G23 was collected from a generally temperate region, while G45 was collected from a region that is typically dry), and the difference in extraction efficiencies between genotypes most likely arises from the geographical adaptation of these plants. The methanol (X-M) protocol typically yielded more metabolites, though the change was not always statistically significant. Besides the plant cell-wall structure being specific to each genotype, one reason for the difference in extraction efficiency might be that, during the X-MC protocol, some polar metabolites might be lost at lower layers (e.g., the chloroform layer). Despite higher metabolite recovery in the X-M protocol, it is also worth noting that population variability within a selected

genotype, monitored as standard deviation, was always found to be larger in the X-M protocol (Figures 2 and 3). One possible reason for this is that the X-M protocol yields two layers (soluble and insoluble) where a large number of metabolites mix in the soluble phase, including chlorophyll (resulting in a greenish mixture as the aliquot), while the X-MC protocol results in three layers (methanol/chloroform and insoluble) and there is better fractionation in metabolites. In that sense, the X-MC protocol is "cleaner" (e.g., chlorophyll is in the middle phase, leaving a clear aliquot in the upper phase).

A first increasing then saturating trend for ATP levels depending on the recovery steps was observed for both genotypes used in our study (Figure 1D), indicating that at least the third recovery step (sp123 in the figure) is required for efficient recovery of metabolite collection and the fourth recovery step might be preferably collected depending on the metabolite of interest, e.g., secondary metabolites that are present in low amounts in the cell. Successive steps can be useful to obtain larger amounts of metabolite for such a study.

The decrease in ATP levels of drought-treated *Brachypodium* individuals (with the X-M protocol) indicated a pronounced effect of drought on energy metabolism. In line with Zagdańska (1995), it can be speculated that these reductions and energetic costs might depend on the slowdown of energy metabolism to decrease the rate of the energy-consuming pathways that do not carry primary importance for survival so that it can maintain cellular integrity due to the water-deficit conditions.

For starch level, typically a decrease is expected upon drought treatment, the reason being the hydrolysis of starch into free glucose under drought conditions for a quick energy source. In our experiments, starch content in leaves (g/g FW) under normal conditions ranged between 0.8%–2.7%, in line with the results of a study that was carried out on the deposition and characterization of starch in *Brachypodium distachyon* (Tanackovic et al., 2014). However, the statistical analysis of estimated starch concentrations showed that there was no significant effect of drought stress on starch content (Figure 4), despite occasional decrease in starch level for some of the individual populations (data not shown), which in turn can be considered within biological variation. One reason for this might be that the starting starch level (background level) is already high, such that we cannot observe significant changes in starch levels. More severe drought treatments might be a solution for this, though this results in pronounced secondary effects. As for glucose, there was a general increase upon drought treatment (Figure 3), in line with our expectations: under stress, glucose mobilized from starch provides a quick energy source. This 1–4-fold

increase in glucose levels is consistent with the literature, where it has been shown that under drought conditions plants accumulate simple sugars such as glucose and sucrose, increasing invertase activity in their leaves (Tuteja, 2010), and increase in glucose concentrations might be up to 5-fold under drought stress (De Roover et al., 2000). Further speculation on which part of the metabolism is affected upon drought treatment needs more high-coverage metabolome data.

Taken together, 2 different extraction protocols, the effect of drought stress, and genotypic differences based on three reporter metabolites in model plant *Brachypodium distachyon* were evaluated. Based on the different response of ATP levels according to extraction method and validation of ATP measurements with spiking experiments, it can be concluded that there is no single protocol that fits all species or even genotypes, and we conclude that tailor-made protocols are needed in plant metabolomics studies. In response to drought stress, the response level differs among genotypes, highlighting the importance of natural variation in *Brachypodium* (Opanowicz et al., 2008; Bourguiba et al., 2012; Verelst et al., 2013). This study will provide a basis for the construction of high-coverage metabolome platforms, and potentially lead to more reliable metabolome studies in plants and help further metabolomics studies on drought stress response in order to improve agriculturally important crop species.

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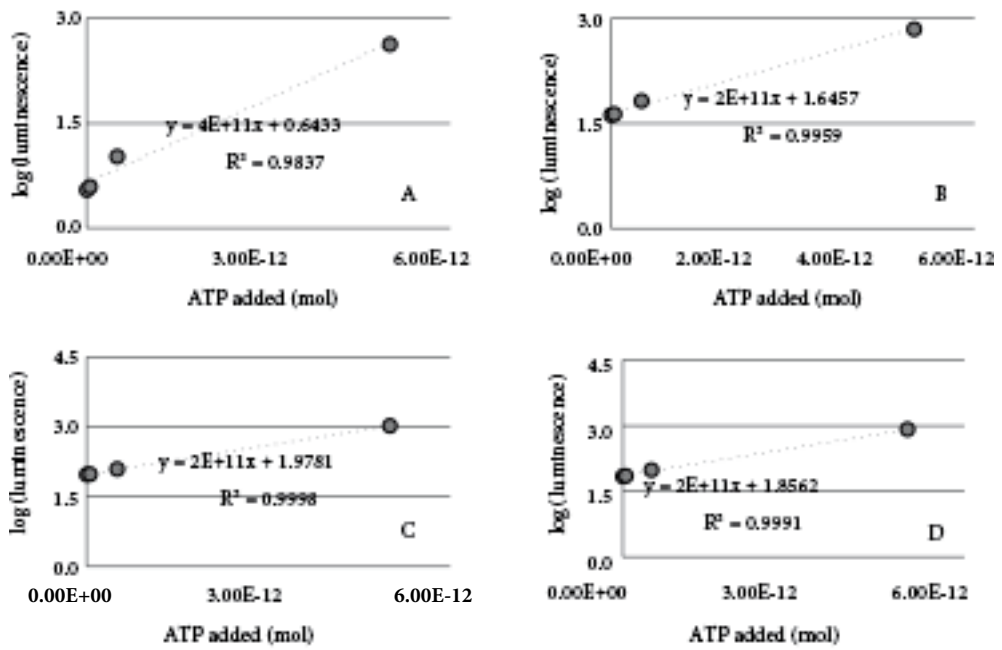
Supplementary Figure 1. Calibration curves with spiked samples: A) G23_1, B) G23_2, C) G45_1, D) G45_2.