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Metabolite Analyses of Grain from Maize Hybrids Grown in the United States under Drought and Watered Conditions during the 2002 Field Season

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Understanding natural variation in the composition of conventional crop germplasms is critical in establishing a baseline for comparison of biotechnology-derived crops. This is particularly relevant to such traits as tolerance to drought stress. Thus, there is both a need to understand the contribution of stress conditions to natural variation in plant nutritional components and to determine whether levels of small molecule metabolites such as osmoprotectants and stress metabolites are also affected. As a first step in developing such information for maize, seven conventional hybrids were grown under different moisture regimens and the impact of moisture on composition was assessed. The regimens included well-watered conditions, water restriction during the vegetative phase, and water restriction during grain fill. Compositional analyses of the harvested grain included assessments of the levels of proximates (moisture, protein, oil, starch) and small molecule metabolites such as fatty acids, free amino acids, organic acids, sugars, total glycerol, glycine betaine, and abscisic acid. Ranges for these analytes were determined across all moisture regimens, and the effect of the different water regimens on these analytes was also evaluated. The number and type of grain analytes that showed statistically significant differences in levels between different water regimens differed quite markedly by maize hybrid. However, the magnitude of mean differences between well-watered and water-restricted samples was typically small, and statistically significant differences for any given analyte were typically observed in only one to three of the seven maize hybrids. Only two analytes, free glutamine and free proline, showed a significant drought-induced difference in at least four maize hybrids.

KEYWORDS: Maize; *Zea mays* L.; compositional variation; metabolic profiling; drought

INTRODUCTION

Compositional evaluations are conducted as a key component of the safety assessment of crops developed through modern biotechnology to assess potential unanticipated changes that may arise during either plant transformation and regeneration or expression of the inserted genes (*1*−*9*). A list of well-defined metabolites for assessment in compositional studies for new biotechnology crops has been developed by the Organization for Economic Co-operation and Development (OECD) (*5*−*8*). The principles and analytes of the OECD consensus are well-accepted globally and are generally consistent with data requirements in the United States, Canada, the European Union, and other countries. The International Life Sciences Institute (ILSI) now maintains a database that has accumulated extensive compositional data on key crops such as maize, soybean, cotton, and canola, based on the analytes recommended by OECD (*10*). However, the crops annotated in the ILSI crop composition database were grown under "normal agricultural conditions" and may not have been exposed to the full range of natural variation that would be encountered during environmental stress conditions, such as water deficit. Furthermore, additional analytes may need to be considered for evaluation depending on the

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intended trait or change in the crop that has been modified by modern biotechnology. This is particularly true (i) when traits for which significant differences in metabolite composition are intended, such as increases in the levels of essential minerals or vitamins, are addressed or (ii) when the crop is modified to enhance a complex trait such as tolerance to abiotic stress. As part of an earlier study (*11*) to assess natural compositional variation in grain harvested from a diverse genetic range of maize lines we measured selected osmoprotectants and stress metabolites, as well as other small molecule metabolites that may reasonably be expected to change in response to moisture availability. Increases in the levels of osmoprotectants and compatible solutes (such as sugars, polyols, and certain quaternary ammonium compounds) have been shown to represent a nearly universal adaptation or response to induced stress and have been observed in a range of cells, tissues, crops, and other organisms (*12*, *13*). They do not appear to interfere with normal metabolism and act through antioxidant properties or by stabilizing proteins (*12*−*14*). Secondary metabolites such as salicylic acid (*15*) and abscisic acid (*16*) are also known to be associated with stress in many plant tissues. The study was therefore designed not only to assess the effect of water restriction on variation in levels of nutritional components such as oil, protein, and starch in maize grain but as a means to determine any impact on small molecule metabolites including those implicated as osmoprotectants and stress metabolites. The compositional analysis centers on maize grain collected from conventional lines grown in the field under three different moisture regimens: (i) well-watered, (ii) water-restricted during the vegetative phase, and (iii) water-restricted during grain fill. A total of seven conventional maize hybrids were studied.

MATERIALS AND METHODS

Biological Material. Seeds of the seven maize (*Zea mays* L.) hybrids were planted at Wichita, KS, in the summer of 2002. The hybrids included the following commercially available lines: DK539, DK595, and RX670 as well as four testing pedigrees referred to herein as experimental hybrids 1, 2, 3, and 4. A randomized complete block design with four replications of each of three watering treatments was used. During the growing season, approximately 13 in. of rainfall was observed at this field location. A Zimmatic lateral-move irrigator (Lindsay Corp., Omaha, NE) fitted with individually valved spray nozzle drops on 5 ft centers was used for application of the irrigation water. A suitable buffer zone was planted between treatments to allow isolation of each water treatment within and between replications. Water was withheld by manually closing the boom drop valves while the spray head was traveling over the area to be droughted. Normal rainfall was sufficient to prevent drought conditions up to 2 weeks prior to anthesis. In this time frame, the grain fill drought treatment (FillDry) and control plants were given three irrigations of 0.5 in. that was withheld from the vegetative dry treatments. This deficit caused a 10−20% reduction in the rate of plant height growth. All treatments were given three 1 in. waterings during the week of anthesis, July 11−19. During the 3 weeks following anthesis, plants exposed to drought during the vegetative phase (VegDry) and the control plants were given three 0.8 in. waterings that were withheld from the grain fill drought treatment. Yield losses combined across all seven maize hybrids yield were approximately 20% in both water restriction treatment regimens relative to that of control. Contributing factors to yield loss included decreased kernels per ear and decreased average kernel and number of plants per acre. Net yield reduction for the maize hybrids could be attributed to different ear yield component changes. Overall, the VegDry phase decreased the number of kernels per ear, whereas the FillDry phase decreased average kernel mass.

Near-Infrared Transmission (NIT) Analysis of Proximates. Moisture, oil, protein, and starch were determined on an Infratec 1220 series grain analyzer (Foss North America, Eden Prairie, MN).

Measurement of Fatty Acids. Aliquots of oil (20−60 mg) derived from Foss Tecator 2050 extraction were used for fatty acid analysis as described previously (*11*). Fatty acid methyl esters were formed by trans-esterification of the extracted oil with acetyl chloride/methanol at ambient temperature with constant agitation overnight (16−24 h). The fatty acid methyl esters were extracted into hexane and analyzed by capillary gas chromatography with a flame ionization detector (HP 6890 series, Agilent). A 19 min temperature run (initial, 170 °C for 10 min, increased to 240 °C at 10 °C/min, held for 2 min) on a Supelco Omegawax 320 fused silica capillary column (30 m × 0.32 mm × 0.25 μm film thickness) was used to separate the fatty acid methyl esters in order of increasing carbon chain length (from C10 to C24). The instrument detection limits were 2.5 μg/mL. Data were recorded as area percent of fatty acid composition. Fatty acid methyl ester standards were purchased from Sigma-Aldrich (St. Louis, MO) or Matreya (Pleasant Gap, PA).

Measurement of Free Amino Acids, Sugars, Organic Acids, Abscisic Acid, Glycine Betaine, and Total Glycerol. All methods used in assessments of the levels of these metabolites have been described previously (*11*).

Statistical Analyses. The purpose of the statistical analysis was to assess treatment (i.e., water-fed condition) differences for each analyte across maize hybrid and also within individual maize hybrids. For the across maize hybrid analysis, a model of the following form was fit for each analyte

$$y_{ijk} = \mu + b_i + s_j + t_k + (st)_{jk} + \epsilon_{ijk} \quad (1)$$

where y_{ijk} is the response for the ith block of the jth maize hybrid and the kth treatment (i.e., FillDry, VegDry, and Wet), μ is the overall mean, b_i is the random effect of the ith block, s_j is the effect of the jth maize hybrid, t_k is the effect of the kth treatment, $(st)_{jk}$ is the effect of the interaction between the jth maize hybrid and the kth treatment, and ϵ_{ijk} is the random error.

To satisfy the model assumptions for glutamine, glycine betaine, histidine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, valine, malate, phenylpyruvate, saccharopine, sorbitol, and stachyose, the analysis was performed on the log(e) transformed data for these analytes. To satisfy the model assumptions for abscicic acid, the analysis was performed on the square root transformed data for this analyte. The model assumptions were satisfied for the remaining analytes.

An outlier analysis was conducted using SAS PROC GLM [SAS Software release 9.1 (TS1M3); copyright 2002−2003 by SAS Institute Inc., Cary, NC] and observations with studentized residuals that were ≤ -6 or ≥ 6 were removed from the analysis dataset. Observations dropped as a result of the outlier analysis included one asparagine value and one glutamine value for experimental hybrid 1 and one glycine value for DK539. The final analysis dataset consists of the transformations described above and the removal of the observations deemed to be outliers. On the final analysis dataset, SAS PROC MEANS was used to calculate the sample mean, range, and standard error for each analyte and treatment across maize hybrid.

Model 1 was fit for each analyte using SAS PROC MIXED to test for significant differences among the three treatments across maize hybrids. As noted in the relevant table footnotes, if the interaction between maize hybrid and treatments is significant, then the treatment main effect should not be interpreted. If the interaction is not significant, then the treatment main effect may be assessed for statistical significance. All statistical comparisons are made at the 5% level of significance (i.e., $p < 0.05$).

For the within-hybrid analysis, the data were analyzed by maize hybrid with a separate analysis performed for each analyte. A model of the following form was fit for each maize hybrid and analyte

$$y_{ij} = \mu + b_i + t_j + \epsilon_{ij} \quad (2)$$

where y_{ij} is the response for the ith block and the jth treatment (i.e., FillDry, VegDry, and Wet), μ is the overall mean, b_i is the random effect of the ith block, t_j is the effect of the jth treatment, and ϵ_{ij} is the random error.

Table 1. Summary of Values and Significant Differences in Proximates across All Maize Hybrids

component[a]	FillDry mean (range)[b]	VegDry mean (range)[b]	Wet mean (range)[b]	hybrid *p* value	treatment *p* value	interaction *p* value	lit. range	ILSI[d] mean (range)
moisture[e]	7.79 (7.20–8.50)	7.90 (7.30–8.60)	8.08 (7.40–9.40)	0.0040	0.0145	0.8981	7–23[f] 8.2–26.2[g]	11.2 (6.1–26.2)
oil[e]	3.96 (3.19–4.35)	4.14 (3.58–4.85)	4.23 (3.85–4.87)	0.0001	0.0001	0.2479	3.1–5.7[f] 2.48–4.81[g]	3.55 (1.74–5.56)
protein[e]	10.08 (8.30–11.60)	10.84 (7.80–14.10)	9.68 (7.90–11.20)	0.3190	0.0005	0.8521	6–12[f] 9.7–16.1[h]	10.25 (6.15–15.01)
starch[e]	69.64 (68.50–70.50)	69.42 (68.50–70.60)	69.92 (68.90–71.00)	<0.0001	0.0006	0.2755	77.4–87.2[g] 82.2–88.1[i]	84.7 (77.4–89.5)

[a] Percent dry weight except for moisture. [b] Range denotes the lowest to the highest individual values across all maize hybrids. [c] If the interaction between the effects of germplasm and treatment is significant, then the germplasm and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the germplasm/hybrid and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [d] ILSI database, 2006 (*10*). [e] Measurements were by NIT. [f] Watson, 1982 (*17*). [g] Sidhu et al., 2000 (*18*). [h] Jugenheimer, 1976 (*19*). [i] Ridley et al., 2002 (*20*).

Table 2. Summary of Values and Significant Differences in Fatty Acids across All Maize Hybrids

component[a]	FillDry mean (range)[b]	VegDry mean (range)[b]	Wet mean (range)[b]	hybrid *p* value	treatment *p* value	interaction *p* value	lit. range	ILSI[d] mean (range)
C16:0 palmitic acid	16.08 (13.50–17.70)	15.46 (12.80–18.00)	15.93 (13.50–18.90)			0.0288	7–19[e]	11.50 (7.94–20.71)
C18:0 stearic acid	2.03 (1.60–2.50)	1.94 (1.50–2.50)	2.04 (1.50–2.40)	<0.0001	0.0074	0.5175	1–3[e]	1.82 (1.02–3.40)
C18:1 oleic acid	21.02 (16.50–27.00)	22.02 (17.70–31.80)	22.29 (17.30–29.80)	<0.0001	0.0035	0.0743	20–46[e]	25.80 (17.40–40.20)
C18:2 linoleic acid	59.38 (53.00–64.10)	59.22 (49.80–63.60)	58.30 (51.70–62.90)			0.0177	35–70[e]	57.60 (36.20–66.50)
C18:3 linolenic acid	1.06 (0.80–1.30)	0.96 (0.70–1.20)	1.03 (0.70–1.30)	<0.0001	0.0014	0.3903	0.8–2[e]	1.20 (0.57–2.25)
C20:0 arachidic acid	0.41 (0–0.60)	0.38 (0–0.60)	0.38 (0–0.60)	0.5249	0.4213	0.2049	0.1–2[e]	0.41 (0.28–0.97)

[a] Values of fatty acids expressed as percent of total fatty acid. The method included the analysis of the following fatty acids, which were not detected in the majority of samples analyzed: 12:0 lauric acid, 14:0 myristic acid, 16:1 palmitoleic acid, 22:0 behenic acid, 22:1 ecosenoic acid, and 24:0 lignoceric acid. [b] Range denotes the lowest to the highest individual values across all maize hybrids. [c] If the interaction between the effects of germplasm and treatment is significant, then the germplasm and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the germplasm/hybrid and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [d] ILSI database, 2006 (*10*). [e] Watson, 1982 (*21*).

On the final analysis dataset, SAS PROC MEANS was used to calculate the sample mean, range, and standard error for each analyte, treatment, and maize hybrid. Model 2 was fit for each analyte and maize hybrid using SAS PROC MIXED to test for significant differences among the three treatments.

RESULTS AND DISCUSSION

Proximate Composition. Proximates, as measured by near-infrared transmission spectroscopy (NIT), all showed a statistically significant treatment effect when combined across all maize hybrids (**Table 1**). Mean values for moisture, oil, and starch levels were slightly reduced in the water-restricted treatments, whereas protein levels showed a modest increase. When the effects of water restriction on each individual maize hybrid were compared, statistically significant differences ($p < 0.05$) were typically observed only for a minority of the maize hybrids: two for oil (experimental hybrid 3, RX670), two for starch (experimental hybrids 2 and 3), and none for moisture or protein. Mean treatment differences were typically small, never exceeding 12% for any given analyte compared within a maize hybrid (data not shown). The ranges of values for moisture, oil, and protein were consistent with that recorded in the ILSI Crop Composition Database (*10*) and in the literature (*17*−*20*). Starch values were somewhat different, reflecting alternative methodologies in their measurement. The results imply that water restriction can affect levels of nutrients in maize but that the levels are within the natural ranges recorded for maize.

Fatty Acid Composition. Twelve fatty acids were measured in this analysis. Fatty acids that were above the levels of quantitation included 16:0 palmitic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, 18:3 linolenic acid, and 20:0 arachidic acid. Not detected above their levels of quantitation were 12:0 lauric acid, 14:0 myristic acid, 16:1 palmitoleic acid, 22:0 behenic acid, 22:1 ecoisenoic acid, and 24:0 lignoceric acid; these analytes are known to be present at only very low levels in grain (*9*, *10*, *21*). Of the measured fatty acids 16:0 palmitic acid and 18:2 linoleic acid showed a significant interaction between germplasm and treatment. Of those analytes that did not exhibit an interaction, 18:0 stearic acid, 18:1 oleic acid, and 18:3 linolenic acid showed a treatment effect when compared across all maize hybrids (**Table 2**). When individual maize hybrids were considered, no statistically significant differences were recorded for these fatty acids, although a general trend for lower values in the VegDry treatment was observed (data not shown). The ranges of values for the detected fatty acids reported in this study (**Table 2**) were consistent with those in the ILSI Crop Composition Database (*10*) and in the literature (*21*).

Free Amino Acid Composition. A total of 19 free amino acids were measured in this analysis. Levels were typically low

Table 3. Summary of Significant Differences ($p < 0.05$) in Levels of Free Amino Acids for Each Maize Hybrid

hybrid/ analyte[a]	FillDry mean (range)	VegDry mean (range)	Wet mean (range)	treatment *p* value
DK539				
alanine	157.59 (105.83–184.27)	214.56 (158.52–255.16)	139.97 (107.49–151.68)	0.0010
arginine	126.51 (102.59–144.25)	174.46 (110.75–217.16)	122.93 (109.66–147.35)	0.0310
glutamine	25.96 (18.36–35.79)	117.24 (44.52–175.14)	23.55 (16.29–34.67)	0.0013
glycine	18.11 (14.04–20.47)	45.12 (41.26–49.73)	17.87 (16.25–20.52)	0.0001
isoleucine	18.92 (14.04–21.69)	34.39 (20.63–46.49)	16.25 (14.08–18.42)	0.0125
leucine[b]	14.87 (8.64–19.48)	67.41 (22.80–107.03)	13.27 (10.83–19.50)	0.0018
lysine[b]	46.77 (35.64–54.23)	60.14 (43.43–70.58)	42.51 (39.09–49.84)	0.0142
phenylalanine[b]	16.22 (10.80–19.48)	50.36 (15.20–72.43)	13.81 (10.83–20.59)	0.0085
serine[b]	49.47 (32.40–65.08)	68.80 (51.03–83.24)	38.99 (30.40–48.75)	0.0042
threonine[b]	23.25 (14.04–30.37)	39.27 (24.97–48.65)	20.03 (14.12–24.92)	0.0007
tyrosine[b]	50.81 (38.88–58.19)	109.13 (57.55–142.70)	49.55 (44.52–57.42)	0.0032
DK595				
asparagine	442.77 (334.42–531.32)	340.87 (273.32–530.24)	278.61 (251.09–311.02)	0.0499
aspartic acid	341.99 (262.99–401.73)	225.90 (200.00–271.06)	229.56 (212.74–258.49)	0.0165
histidine[b]	68.13 (56.28–76.67)	56.60 (47.93–72.35)	49.77 (45.65–54.76)	0.0480
methionine[b]	6.76 (4.32–9.76)	15.97 (7.60–25.95)	5.96 (4.33–7.58)	0.0112
proline	510.78 (355.29–621.21)	728.89 (556.64–916.49)	816.00 (779.11–865.28)	0.0146
RX670				
aspartic acid	380.97 (314.94–424.84)	267.37 (227.92–303.26)	291.86 (247.26–342.02)	0.0130
glutamine[b]	16.03 (10.89–21.76)	40.62 (32.72–50.33)	27.27 (16.29–40.48)	0.0240
proline	456.73 (271.24–653.26)	675.93 (543.08–775.60)	644.09 (590.66–688.38)	0.0414
experimental hybrid 1				
proline	357.13 (308.70–394.14)	819.18 (714.75–1003.24)	649.33 (493.99–752.76)	0.0021
experimental hybrid 2				
glutamic acid	350.37 (310.38–403.03)	301.34 (271.74–362.05)	270.13 (225.84–328.99)	0.0061
glutamine[b]	25.00 (17.30–30.50)	37.72 (30.24–53.44)	25.51 (21.72–32.57)	0.0321
histidine[b]	68.15 (53.55–81.26)	60.48 (52.97–71.97)	50.22 (46.69–53.44)	0.0236
leucine[b]	12.76 (9.84–16.25)	11.66 (9.78–13.09)	8.96 (8.65–9.777)	0.0228
phenylalanine[b]	14.14 (10.81–19.67)	10.04 (8.70–12.00)	7.87 (6.51–8.69)	0.0208
experimental hybrid 3				
alanine	128.08 (108.46–165.95)	220.00 (198.70–241.30)	141.11 (133.41–152.17)	0.0166
asparagine	290.56 (203.26–360.99)	479.68 (390.88–568.48)	240.62 (211.33–282.61)	0.0352
aspartic acid	197.74 (158.70–226.93)	280.30 (245.39–315.22)	186.77 (176.47–198.91)	0.0297
glutamine[b]	14.36 (11.93–16.16)	66.27 (60.80–71.74)	29.91 (18.44–55.43)	0.0150
leucine[b]	7.86 (7.59–8.62)	25.54 (14.12–39.96)	8.97 (8.68–9.78)	0.0209
lysine[b]	27.63 (23.91–32.33)	49.98 (35.83–64.13)	25.02 (21.69–31.59)	0.0483
methionine[b]	3.79 (3.25–4.35)	8.15 (6.51–9.78)	4.62 (3.25–5.45)	0.0021
phenylalanine[b]	8.94 (7.61–9.76)	23.91 (14.12–33.70)	8.70 (7.59–9.80)	0.0260
proline	322.36 (248.91–480.60)	993.43 (895.65–1091.21)	616.19 (455.43–850.76)	0.0061
serine[b]	27.09 (24.97–28.26)	49.98 (39.09–60.87)	30.45 (29.20–32.61)	0.0144
threonine[b]	10.57 (9.78–10.86)	23.90 (18.46–29.35)	11.96 (10.85–13.07)	0.0048
tryptophan	9.21 (8.62–9.78)	15.75 13.03–18.48)	9.79 (8.68–11.98)	0.0266
valine[b]	28.44 (27.14–31.25)	57.59 (41.26–73.91)	31.54 (27.11–37.04)	0.0307

[a] Parts per million dry weight. [b] Statistical analysis was performed on the log(*e*) transformed data for this analyte.

and often close to the levels of quantitation (see ref *11*). The major free amino acids as determined in this study were asparagine, aspartate, glutamate, and proline, consistent with previous literature reports (*11*, *22*, *23*). The impact of water restriction on the levels of free amino acids in maize highlighted extensive hybrid dependence, and the total number of free amino acids susceptible to a significant treatment effect differed according to maize hybrid (**Table 3**). Experimental hybrid 3 showed statistically significant treatment differences ($p < 0.05$) in 13 of the measured amino acids, DK539 in 11, experimental hybrid 2 in 5, DK595 in 5, RX670 in 3, experimental hybrid 1 in 1, and experimental hybrid 4 (not listed in **Table 3**) in none. A comparison across all maize hybrids (**Table 4**) showed a significant interaction between germplasm and treatments ($p < 0.05$) in mean values for asparagine, aspartic acid, glycine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and tyrosine. For those free amino acids that did not exhibit a significant interaction effect, a significant treatment effect was observed for alanine, arginine, glutamine, isoleucine, lysine, and valine (generally increased during VegDry) and for glutamic acid and histidine (generally increased during VegDry and FillDry). However, when each maize hybrid was assessed individually, differences in alanine, histidine, and lysine were observed for only two maize hybrids and in arginine and valine for only one (**Table 3**). Only two amino acids, glutamine and proline, showed a significant treatment effect in at least four maize hybrids. Proline is well recognized as a compatible solute and osmoprotectant and as an important component in the stress response of plants. Studies on proline accumulation in maize seedlings (*24*) and roots (*25*) have been reported, although there is little information on free proline levels in kernels. There is at least one report on transcriptomic changes in maize kernel in response to water stress (*26*). The results here indicate that free proline levels are significantly affected by water deficit in at least some maize hybrids.

As a general rule, levels of most free amino acids were highest in the VegDry state; however, there was also substantial overlap in the range of recorded values and marked differences in the mean values for the different maize hybrids (**Table 3**).

Sugar Composition. Simple sugars, with the exception of the antinutrient raffinose, are rarely measured in mature grain harvested from maize. In this study, a comparative screening approach was adopted to facilitate rapid measurement of the large sample set and because semiquantitative data were deemed to be adequate for the statistical analyses. The LC-MS/MS method adopted here allows relative measurements of sucrose, glucose, fructose, raffinose, stachyose, inositol, mannitol/sorbitol, and trehalose (*11*). Sucrose was by far the most abundant of these metabolites. Stachyose, inositol, and mannitol/sorbitol were very close to the limits of detection, however, and trehalose was not detected by this method. These observations are consistent with data previously reported (*11*).

Table 4. Summary of Values and Significant Differences in Free Amino Acids across All Maize Hybrids

component[a]	FillDry mean (range)[b]	VegDry mean (range)[b]	Wet mean (range)[b]	hybrid *p* value	treatment *p* value	interaction *p* value[c]	lit. range
alanine	150.8 (105.8–207.3)	179.15 (95.24–343.51)	146.55 (106.41–200.22)	0.4630	0.0013	0.0859	56–134[d] 34–206[e]
arginine	89.25 (53.26–149.51)	110.46 (58.25–217.16)	85.00 (53.55–147.35)	<0.0001	0.0002	0.1458	26–74[d] 38–172[e]
asparagine	325.34 (189.60–637.05)	314.55 (188.31–568.48)	249.41 (143.32–341.33)			0.0020	185–272[d] 129–617[e]
aspartic acid	278.04 (158.70–424.84)	243.84 (133.1–389.31)	231.77 (154.59–342.02)			0.0002	113–173[d] 138–462[e]
glutamic acid	318.63 (226.09–467.67)	325.13 (249.18–466.81)	284.61 (224.76–374.59)	0.0025	0.0004	0.0509	216–289[d] 202–572[e]
glutamine[f]	21.21 (10.89–40.13)	59.19 (15.30–175.14)	28.83 (12.02–57.73)	0.0010	<0.0001	0.0740	29–141[d] 20–160[e]
glycine	17.35 (11.96–23.84)	22.34 (14.02–49.73)	16.08 (11.96–21.79)			<0.0001	12–56[d] 12–50[e]
histidine[f]	60.80 (45.55–81.90)	61.76 (42.21–100.33)	52.81 (41.21–68.40)	0.0538	0.0005	0.1676	20–30[d] 14–69[e]
isoleucine	17.23 (0–32.50)	21.76 (13.07–46.49)	16.52 (11.96–25.16)	0.0316	0.0056	0.0890	8–18[d] 8–34[e]
leucine[f]	12.49 (7.59–22.75)	24.35 (8.71–107.03)	11.31 (8.65–23.18)			<0.0001	7–20[d] 7–43[e]
lysine[f]	35.74 (22.80–56.34)	43.72 (25.89–70.58)	32.86 (21.69–49.84)	<0.0001	<0.0001	0.1688	13–47[d] 21–250[e]
methionine[f]	5.99 (3.23–11.93)	8.73 (4.33–25.95)	6.45 (3.25–14.22)			0.0203	11–13[d] 0–19[e]
phenylalanine[f]	13.86 (7.61–21.67)	20.27 (8.70–72.43)	10.76 (6.51–20.59)			0.0003	7–37[d] 5–35[e]
proline	450.53 (248.91–802.60)	725.33 (472.94–1091.21)	656.16 (451.68–865.28)			0.0070	137–1127[e]
serine[f]	40.76 (24.97–65.08)	50.12 (32.43–102.51)	39.06 (28.20–66.74)			0.0041	29–90[d] 24–100[e]
threonine[f]	17.59 (9.78–31.42)	22.32 (11.90–48.65)	16.17 (10.85–26.26)			0.0010	10–22[d] 9–42[e]
tryptophan	11.33 (7.57–16.25)	13.33 (8.72–23.78)	11.07 (7.60–15.17)			0.0071	7–15[d] 0–24[e]
tyrosine[f]	49.56 (35.83–68.26)	62.39 (35.71–142.70)	46.90 (39.13–67.32)			<0.0001	28–40[d] 30–91[e]
valine[f]	38.79 (24.97–93.28)	48.95 (30.30–101.42)	34.96 (25.14–56.34)	0.0013	0.0002	0.1717	23–43[d] 16–59[e]

[a] Parts per million dry weight. [b] Range denotes the lowest to the highest individual values across all maize hybrids. [c] If the interaction between the effects of germplasm and treatment is significant, then the germplasm and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the germplasm/hybrid and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [d] Huang et al., 2005 (*22*, *23*). [e] Harrigan et al., 2007 (*11*). [f] Statistical analysis was performed on the log(*e*) transformed data for these analytes.

Table 5. Summary of Values and Significant Differences in Sugars across All Maize Hybrids

analyte[a]	FillDry mean (range)[b]	VegDry mean (range)[b]	Wet mean (range)[b]	hybrid *p* value	treatment *p* value	interaction *p* value[c]	ILSI mean (range)
sucrose	25.88 (18.03–33.27)	24.90 (10.75–33.10)	26.82 (20.11–33.59)	0.1043	0.2059	0.8862	
fructose	1.11 (0.65–1.80)	1.22 (0.59–2.77)	1.27 (0.65–1.97)	0.1887	0.3026	0.7804	
glucose	1.79 (0.98–3.39)	2.49 (0.93–5.46)	2.18 (1.09–3.64)	0.1864	0.0248	0.2232	
sorbitol/mannitol[d]	1.04 (0.50–2.77)	1.09 (0.50–2.05)	1.14 (0.43–1.76)	0.0013	0.3150	0.1423	
inositol	0.034 (0.018–0.051)	0.025 (0.014–0.039)	0.029 (0.016-.0040)	0.0316	<0.0001	0.6029	1469.1[e] (1236.0–2009.5)[e]
raffinose	1.64 (0.92–2.23)	1.51 (0.67–2.20)	1.38 (0.86–2.12)	<0.0001	0.0015	0.6378	0.142[f] (0.056–0.290)[f]
stachyose[d]	0.031 (0.016–0.057)	0.030 (0.015–0.068)	0.033 (0.014–0.10)	0.726	0.9113	0.8140	

[a] Response units/fresh weight. [b] Range denotes the lowest to the highest individual values across all maize hybrids. [c] If the interaction between the effects of germplasm and treatment is significant, then the germplasm and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the germplasm/hybrid and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [d] Statistical analysis was performed on the log(*e*) transformed data for these analytes. [e] Parts per million dry weight (*10*). [f] Percent dry weight (*10*).

Of the sugars tested, glucose, raffinose, and inositol showed a treatment effect when calculated across all maize hybrids (**Table 5**). However, mean values for inositol levels in all treatments were very close to the limits of quantitation, and these data should be interpreted with caution. Mean levels for glucose were typically highest in the VegDry samples and lowest

Table 6. Summary of Values and Significant Differences in Organic Acids across All Maize Hybrids

analyte[a]	FillDry mean (range)[b]	VegDry mean (range)[b]	Wet mean (range)[b]	hybrid *p* value	treatment *p* value	interaction *p* value[c]
citric acid	44.49 (23.33–59.66)	41.82 (26.31–75.49)	39.68 (23.99–54.27)			0.0438
fumaric acid	0.18 (0.074–0.37)	0.19 (0–0.35)	0.16 (0.077–0.24)			<0.0001
glutaric acid	0.032 (0.026–0.080)	0.042 (0.026–0.080)	0.036 (0.021–0.071)	<0.0001	0.0619	0.1152
isocitric acid	0.20 (0.12–0.35)	0.19 (0.031–0.34)	0.19 (0.10–0.29)	<0.0001	0.4627	0.7304
malic acid[d]	4.47 (1.60–7.72)	3.10 (1.33–4.86)	3.28 (1.33–5.27)			0.0037
phenylpyruvic acid[d]	0.067 (0.011–1.00)	0.081 (0.0093–0.58)	0.039 (0.0078–0.26)	0.6987	0.4016	0.8934
saccharopine[d]	0.058 (0.035–0.11)	0.059 (0.034–0.12)	0.048 (0.028–0.083)	0.0127	0.0091	0.3918
succinic acid	0.67 (0.51–1.18)	0.58 (0.43–0.83)	0.61 (0.46–0.81)			0.0002

[a] Response units/fresh weight, the method included analysis of the following organic acids which were not detected; chorismic acid, anthranilic acid, homogentisic acid, α-ketoglutaric acid, phenylpyruvic acid, 4-hydroxyphenylpyruvic acid, prephenic acid, pyruvic acid, salicyclic acid, and shikimic acid. [b] Range denotes the lowest to the highest individual values across all maize hybrids. [c] If the interaction between the effects of germplasm and treatment is significant, then the germplasm and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the germplasm/hybrid and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [d] Statistical analysis was performed on the log(*e*) transformed data for this analyte.

in the FillDry, but statistical significance was observed only once (DK539) when individual maize hybrids were assessed. Mean levels for raffinose were generally higher in both water-restricted regimens when compared to the fully watered regimen but, again, statistical significance was observed only once (experimental hybrid 1) when individual maize hybrids were assessed.

Organic Acid Composition. Organic acids are rarely measured in mature grain harvested from maize. In this study, a comparative screening approach was adopted to facilitate rapid measurement of the large sample set, to maximize organic acid metabolite coverage, and because semiquantitative data were deemed to be adequate for the statistical analyses. The LC-MS/MS method adopted here allows relative responses of the following metabolites: citric acid, succinic acid, malic acid, isocitric acid, fumaric acid, glutaric acid, phenylpyruvic acid, saccharopine, chorismic acid, anthranilic acid, homogentisic acid, α-ketoglutaric acid, 4-hydroxyphenylpyruvic acid, prephenic acid, pyruvic acid, salicyclic acid, and shikimic acid (*11*). Only the first eight in the above list were detected, and, of these, only citric acid, succinic acid, and malic acid had responses that were markedly above the levels of detection. This is somewhat similar to our previous study (*11*), although there we were unable to detect saccharopine or phenylpyruvic acid, which are recorded here.

Of the organic acids tested, a significant interaction between the maize hybrids and treatments was observed for citric acid, fumaric acid, malic acid, and succinic acid when calculated across all maize hybrids (**Table 6**). Of those organic acids that did not exhibit an interaction, only saccharopine, a catabolite of lysine, showed a treatment effect when calculated across all maize hybrids (**Table 6**). Mean levels were increased in both water-restricted treatments (VegDry and FillDry). There were no statistical differences observed when each individual test substance was assessed (results not shown), although the trend to higher values in the water-restricted treatments was maintained. Measured levels were close to the limits of quantitation in all treatment regimens, and these data should be interpreted with caution. Only malic acid (DK539, DK595, experimental hybrid 2), fumaric acid (DK539, experimental hybrids 1 and 3), and citric acid (experimental hybrid 3) showed statistical differences when treatments within individual maize hybrids were considered. For the three maize hybrids cited, malic acid was highest in FillDry and lowest in VegDry. This trend was observed for malic acid in three other maize hybrids, albeit at *p* values of >0.05. Neither fumaric acid nor citric acid showed consistent or meaningful trends between treatments.

Total Glycerol, Glycine Betaine, and Abscisic Acid Composition. Three metabolites considered to be potentially relevant to stress included abscisic acid, glycerol, and glycine betaine. Although abscisic acid has long been proposed to play a role in maize kernel development and levels in leaf are known to increase with water deficit, concentrations of this hormone in all tissues are typically very low (*27*−*31*). This was confirmed in our LC-MS assay, where we found that the concentrations in maize grain from most samples were close to the limit of quantitation (**Table 7**). No treatment effect was observed when combined across all maize hybrids, but a significant treatment effect was observed for experimental hybrid 1.

Free glycerol is often considered to be an important osmolyte but, to our knowledge, has not been measured in mature maize grain. In this study, total glycerol showed a statistically significant treatment effect when calculated across all maize hybrids (**Table 7**). Total glycerol levels were typically higher in the water-restricted samples when compared to the corresponding watered treatment; statistical significance was observed for two maize hybrids (experimental hybrid 3, RX670). Interestingly, whereas the values reported here ranged from 1.13 to 114.74 ppm, our previous survey of conventional lines grown under normal agronomic conditions (*11*) showed extensive variability in total glycerol levels in grain with values ranging from 14.5 to 491.62 ppm.

Glycine betaine is not regularly measured in grain and, to our knowledge, no prior literature is evident. When combined across all maize hybrids, glycine betaine showed a significant treatment × germplasm interaction. Glycine betaine levels were generally higher in the water-restricted samples (**Table 7**). One maize hybrid showed a significant treatment effect (experimental hybrid 3). As an identified osmoprotectant, glycine betaine

Table 7. Summary of Values and Significant Differences in Total Glycerol, Glycine Betaine, and Abscisic Acid across All Maize Hybrids

analyte[a]	FillDry mean (range)[b]	VegDry mean (range)[b]	Wet mean (range)[b]	hybrid *p* value	treatment *p* value	interaction *p* value[c]	lit. range[d]
abscisic acid[e]	0.0020 (0–0.0052)	0.0021 (0–0058)	0.0020 (0–0.0060)	0.1069	0.7768	0.1059	0.0023–0.0058
glycerol	36.40 (1.13–104.76)	49.91 (5.40–114.74)	26.11 (2.34–54.99)	0.0494	0.0011	0.4813	7.99–481.60
glycine betaine	20.44 (2.16–52.21)	18.84 (4.58–58.11)	15.56 (2.61–43.02)			0.0125	1.40–79.20

[a] Parts per million dry weight. [b] Range denotes the lowest to the highest individual values across all maize hybrids. [c] If the interaction between the effects of germplasm and treatment is significant, then the germplasm and treatment effects should not be interpreted. If the interaction between these effects is not significant, then the germplasm/hybrid and treatment effects may be assessed. All statistical comparisons are made at the 5% level of significance ($p < 0.05$). [d] Harrigan et al., 2007 (*11*). [e] Statistical analysis was performed on the square root transformed data for this analyte.

has been well studied in maize leaf (*32*, *33*), and levels are known to increase in growing seasons that experience drought (*34*, *35*).

Conclusion. Seven conventional maize hybrids were grown under different water regimens in the field and compositional analyses conducted on the harvested grain. The purpose was to assess the effect of water restriction on variation in levels of nutritional components in maize, such as protein, oil, and starch, and to include an evaluation of small molecule metabolites including selected osmoprotectants and known stress metabolites, the levels of which might be considered to be susceptible to change upon water restriction. When combined across all maize hybrids, water deficit was seen to affect the levels of many analytes, although only free glutamine and free proline showed a significant treatment effect in at least four individual maize hybrids. Analyte differences that could be attributed to treatment effects when calculated across all maize hybrids, and where no interaction with germplasm was observed, included mean values for moisture, oil, protein, starch, six free amino acids (alanine, arginine, glutamine, histidine, lysine, valine), 18:0 stearic acid, 18:3 linolenic acid, saccharopine, glucose, inositol, raffinose, and total glycerol. Analyte differences that could be attributed to treatment × germplasm interactions included 13 free amino acids, 16:0 palmitic acid, 18:1 oleic acid, 18:2 linoleic acid, citric acid, fumaric acid, malic acid, succinic acid, and glycine betaine.

Changes in the numbers of analytes that showed statistically significant differences between different water regimens differed according to maize hybrid; some maize hybrids were more susceptible to treatment effects than others. This was particularly striking for the free amino acids (see **Table 3**). The finding that for many germplasms water restriction can contribute to substantial variation in free amino acids levels may have implications for compositional analyses of such new crops. Specifically, it implies that these analyses should focus on the introduced trait (or target amino acid) as broad profiling of free amino acids is unlikely to yield data that can be solely defined in terms of the introduced trait. In other words, recorded differences in nontarget amino acids can be attributable to many factors, particularly environmental and climatic.

Our study further revealed that some analytes recognized as osmoprotectants or stress metabolites in other tissues and organisms may be of relevance in compositional studies of stress-exposed maize grain. This is particularly true for proline and total glycerol. Overall, however, although data on the impact of water restriction on grain composition are provided, it is concluded that for most metabolites treatment-induced changes on analytes were modest and not consistent across different germplasms.

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