Physiological and Transcriptional Response to Drought Stress Among Bioenergy Grass Miscanthus Species

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Research

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Abstract

Background: Miscanthus is a commercial lignocellulosic biomass crop owing to its high biomass productivity, particularly in the temperate regions. This study was conducted to elucidate physiological and molecular responses of four *Miscanthus* species subjected to well-watered and droughted greenhouse conditions.

Results: A significant biomass loss was observed under drought conditions for all genotypes. A sterile *M. x giganteus* showed a lower reduction in biomass yield under drought conditions compared to the control than the other species. Under well-watered conditions, biomass yield was as good as or better than control conditions in all species tested. *M. sinensis* was more tolerant than *M. sacchariflorus* to both water stress conditions. 4,389 of the 67,789 genes (6.4%) in the reference genome were differentially expressed among four *Miscanthus* species. Most of the genes were differentially expressed in a single species, but the enrichment analysis of gene ontology (GO) terms revealed that the same biological processes were regulated in all the species during stress conditions. Namely, upregulated differentially expressed genes were significantly involved in sucrose and starch metabolism, redox, and water and glycerol homeostasis and channel activity. Multiple copies of starch metabolic enzymes BAM and waxy GBSS-I were strongly up-regulated in drought stress in all Miscanthus genotypes. Twelve aquaporins (PIP1, PIP2 and NIP2) were also up-regulated in drought stress across genotypes. On the other hand, downregulated differentially expressed genes were significantly involved in protein kinase activity, cell receptor signalling and phosphorylation.

Conclusions: Findings in the present study can assist in implementing molecular breeding approaches of drought resistant *Miscanthus* and its domestication.

Background

The global challenge of feeding the ever-increasing world population is exacerbated due to food crops being used as feedstock for green energy production; in order to minimize carbon footprints. In 2007, a third of corn planted over 92.9 million acres in the USA was used for ethanol production which subsequently caused a 73% increase in corn prices around the world by 2010 [1]. Hence, diverting food crops such as corn, wheat and sugarcane for ethanol production, is causing an unethical ripple effect on food security around the globe. Therefore, alternative ethanol and chemical productions, from plant sources, should prioritise plant species with the following attributes; being no food, perennial, and able to grow on marginal lands, having high biomass yield, low chemical and mechanical input requirement, enhanced water use efficiency and high carbon storage capacity [2, 3, 4]. Amongst grass species, *Miscanthus* species are excellent candidates fulfilling most of the qualities above.

Miscanthus spp. are semi-domesticated rhizomatous perennial C_4 grass species, originally from Eastern Asia [1]. *Miscanthus* species have been used as forage species, in Japan, Korea and China, for thousands of years [5, 6]. Because of its high biomass yield and high ligno-cellulose content, *Miscanthus* spp. are presently being probed as feedstock for bioenergy production [7, 8, 9, 10]. A decade long yield trial in Europe showed that *Miscanthus x giganteus* produced up to 40 tonnes per hectare and year of dry matter, following two years of establishment [11]. A study on its biofuel capacity showed that *Miscanthus* is more efficient in ethanol production per hectare than switch grass and corn [12]. Among the different species, *M. x giganteus*, a sterile triploid hybrid (3n = 57, x = 19), *M. sacchariflorus* (2n = 4x = 76), *M. sinensis* (2n = 2x = 38) and new hybrids between *M. sacchariflorus* and *M. sinensis* are commercially grown as a biomass feedstock [13, 14, 15].

An outstanding photosynthetic capacity at low temperature ca. 5^{o}C makes *Miscanthus* species an ideal biofuel crop for the temperate world [16, 17]. However, yields at many sites in Europe can be limited by insufficient water supply and plant survival is endangered under extreme summer drought [18]. Water scarcity is particularly relevant in Southern Europe where high temperature and irradiation are common. Weng [19] reported differences in osmotic adaptation, among four *Miscanthus* ecotypes, exposed to water stress, and found a relationship between the annual rainfall at a location and the degree of osmotic adaptation. These findings were confirmed by microclimate and geographical modelling which indicated that genotype origin correlated with drought tolerance and explained the different responses observed within the same species [20].

In a greenhouse study on *M. x giganteus*, where water supply was restricted, several physiological responses were observed, reduction in stem elongation rate was the primary response, after induction of abiotic stress and the most significant one [21]. Furthermore, a reduction in photosynthetic performance (chlorophyll content of leaves) and plant water status (leaf relative water content) were also observed during the same experiment. RNAseq analysis with one drought tolerant accession of *M. sinensis* in a time series with six collection time points between zero and 60 days of drought stress revealed that a 15 days period is a threshold to trigger a cascade of responses under water deficit stress [22]. In a pot study under reduced water supply conditions, *M. sacchariflorus* had the highest dry matter per plant, followed by *M. x giganteus* [18]. On the contrary, little is known about the productivity of *Miscanthus* under well-watered and moisture saturated soil conditions commonly experienced on marginal lands.

A few differential expression studies were carried out in *Miscanthus* species to identify transcripts that regulate molecular mechanisms of the plant under different abiotic stress conditions, such as; drought tolerance in *M. sinensis* [22] and salt tolerance in *M. lutarioriparius*: [23, 24]. Our study aims: 1) to elucidate physiological responses in three *Miscanthus* species and a newly bred hybrid in three treatment conditions, and (2) utilise the induced physiological conditions for an in-depth transcriptome study on the molecular basis of water stress in *Miscanthus* species. Our results will contribute to facilitate future genomics assisted breeding in *Miscanthus*.

Results

Phenotypic characterization

In the present study, three *Miscanthus* species and a new hybrid underwent three water treatment conditions and responses of each genotype were evaluated in terms of electrolyte leakage, relative water content (RWC) (at two time points) and biomass fresh and dry weight (Fig. 1).

Electrolyte leakage

Significant effects ($P < 0.05$) on electrolyte leakage (logarithmic value) were observed for all contrasts in the experiment (Table 1). Genotype x treatment interaction effects were significant at $P < 0.001$. When comparing the three treatment conditions, highest mean electrolyte leakage was recorded for all genotypes under well-watered conditions, indicating stress induced by excess water, except for G3 and G4. On the contrary, the least mean electrolyte leakage was recorded for G5 ‘Illinois’ in both control and drought treatment conditions (Fig. 1 and Table 2).

Table 1
Analysis of variance (REML method), fixed effects are displayed for traits electrolyte leakage, relative water content, fresh weight and dry weight

Effects	Electrolyte leakage	Relative water content	Fresh weight	Dry weight
Treatment	0.0204	0.0039	< .0001***	0.0012**
Genotype	0.0061	0.0039	< .0001***	< .0001***
Genotype * treatment	0.0002	0.0005	0.0163*	0.0862 ns
Block	0.0296	0.0819	0.4832 ns	0.2247 ns
Date	NA	0.0165	NA	NA
Date*Genotype	NA	0.5691	NA	NA
Treatment*Genotype* Date	NA	0.0111	NA	NA

◊ NA = not applicable

Table 2
Estimates and confident intervals (brackets) of electrolyte leakage, relative water content, fresh biomass, dry biomass and fresh biomass for six genotypes and three treatments (control, drought, well-watered).

Genotype	Treatment	Electrolyte leakage (25/09/13)	Relative water content T1 (13/09/13)	Relative water content T2 (24/09/13)	Fresh weight -g- (25/09/13)	Dry weight -g- (27/09/13)	RNA-seq libraries
(G1) *M. sacchariflorus* var. Dk-1	Control (C)	6.26 (5.67, 6.90)	96.98 (95.24, 98.53)	98.60 (97.06, 99.97)	62.44 (54.06, 72.08)	12.12 (10.32, 14.21)	M39, M51, M63
	Drought (D)	6.29 (5.50, 7.17)	95.22 (92.61, 97.47)	87.73 (83.87, 91.04)	33.33 (27.05, 41.00)	9.83 (7.75, 12.40)	M35, M47, M59
	Well-watered (W)	7.14 (6.26, 8.13)	97.34 (95.08, 99.28)	98.43 (96.35, 100.22)	49.81 (40.53, 61.17)	10.71 (8.46, 13.49)	M31, M43, M55
(G3) *M. sacchariflorus*	Control (C)	6.24 (5.66, 6.88)	98.65 (97.12, 100.02)	98.07 (96.46, 99.49)	57.02 (49.36, 65.84)	12.16 (10.35, 14.25)	NA
	Drought (D)	4.79 (4.16, 5.49)	95.96 (93.47, 98.10)	95.07 (92.42, 97.33)	31.38 (25.46, 38.62)	7.90 (6.19, 10.02)	NA
	Well-watered (W)	6.23 (5.45, 7.11)	97.76 (95.57, 99.64)	97.59 (95.37, 99.50)	57.43 (46.75, 70.49)	12.00 (9.50, 15.09)	NA
(G2) *M. sinensis* var. genotype-48	Control (C)	6.64 (6.02, 7.31)	98.15 (96.15, 99.57)	98.25 (96.67, 99.66)	50.80 (43.96, 58.67)	9.84 (8.36, 11.57)	M40, M52, M64
	Drought (D)	6.46 (5.65, 7.36)	96.80 (94.44, 98.82)	96.25 (93.81, 98.35)	32.80 (26.62, 40.36)	8.12 (6.37, 10.29)	M36, M48, M60
	Well-watered (W)	6.83 (5.98, 7.78)	97.64 (95.43, 99.54)	96.37 (93.95, 98.45)	71.09 (57.92, 87.21)	13.78 (10.94, 17.29)	M32, M44, M56
(G4) *M. x giganteus*	Control (C)	6.16 (5.58, 6.79)	99.03 (97.54, 100.35)	98.41 (96.85, 99.80)	33.60 (28.72, 39.27)	6.41 (5.40, 7.59)	NA
	Drought (D)	6.71 (5.87, 7.64)	97.11 (94.81, 99.08)	96.70 (94.33, 98.73)	26.48 (21.46, 32.62)	5.79 (4.48, 7.40)	NA

Genotype	Treatment	Electrolyte leakage (25/09/13)	Relative water content T1 (13/09/13)	Relative water content T2 (24/09/13)	Fresh weight -g- (25/09/13)	Dry weight -g- (27/09/13)	RNA-seq libraries
	Well-watered (W)	6.20 (5.42, 7.08)	96.15 (93.69, 98.29)	97.99 (95.84, 99.84)	41.41 (33.66, 50.89)	7.88 (6.17, 10.00)	NA
(G5) *M. x giganteus* cv. Illinois	Control (C)	5.89 (5.33, 6.50)	98.03 (96.42, 99.46)	97.84 (96.21, 99.29)	78.57 (68.07, 90.67)	15.75 (13.45, 18.42)	M41, M53, M65
	Drought (D)	4.27 (3.70, 4.91)	96.50 (93.72, 98.83)	86.09 (81.96, 89.63)	35.54 (28.86, 43.71)	10.86 (8.58, 13.68)	M37, M49, M61
	Well-watered (W)	7.67 (6.73, 8.72)	96.19 (93.73, 98.29)	98.43 (96.35, 100.22)	77.32 (63.01, 94.83)	14.81 (11.77, 18.57)	M33, M45, M57
(G6) *Miscanthus* hybrid '3n'	Control (C)	5.89 (5.33, 6.49)	99.15 (97.68, 100.46)	98.88 (97.38, 100.22)	61.52 (53.27, 71.02)	15.58 (13.30, 18.21)	M42, M54, M66
	Drought (D)	6.17 (5.39, 7.03)	97.76 (94.40, 98.79)	88.80 (84.55, 92.37)	32.68 (26.51, 40.21)	10.53 (8.31, 13.27)	M38, M50, M62
	Well-watered (W)	7.23 (6.34, 8.23)	96.59 (94.20, 98.64)	98.51 (96.44, 100.29)	82.27 (67.05, 100.89)	23.34 (18.66, 29.13)	M34, M46, M58

Relative water content (RWC)

Significant effects ($P < 0.05$) on RWC (logarithmic value), except for block effects and Date-genotype treatment interaction, were observed for all other contrasts in the experiment (Table 1). RWC was recorded at two time points and significant reduction in RWC was observed at the second time point (Fig. 1). No significant difference in RWC was observed between control and well-watered treatment conditions, for most genotypes, at the second time point.

Fresh biomass

Highly significant effects ($P < 0.05$) on fresh biomass (logarithmic value), except for block, were observed for all other contrasts in the experiment (Table 1). Mean fresh biomass was the highest, in well-watered conditions, for all genotypes except for G1 and G5 (Fig. 1 and Table 2). G5 scored the highest mean fresh weight both in control and drought conditions and the second highest in well-watered conditions.

Dry biomass

Highly significant effects ($P < 0.01$) for dry biomass (logarithmic value), except for block and genotype-treatment interaction, were observed for all other contrasts in the experiment (Table 1). As was observed for fresh biomass

weight, dry biomass was significantly reduced under drought conditions. G5 scored the highest mean dry biomass both in control and drought treatment conditions (Fig. 1 and Table 2).

Rna-seq Analysis Under Water Stress

Four genotypes were selected towards the end of the experiment and sequenced in 2014. The number of raw reads from each library range from 16.9M to 42.4M and total of 945.2M reads were obtained for all samples (Suppl. Table S1). After filtering out adaptor sequences and ambiguous and/or low-quality reads, clean reads summed to 926.8M for all samples. Alignment and mapping summary for each library is presented in Suppl. Table S1 and read counts per gene in Suppl. Table S2.

When the normalised counts (Suppl. Table S3) were used to cluster the samples (Fig. 2), these clustered firstly by species (PC1: 30% variance) and later by treatment (PC2: 21% variance). *M. sacchariflorus* and the *Miscanthus* hybrid 3n clustered together and separated from *M. sinensis* and *M. x giganteus*, which clustered together to each other. However, treatment effect was only observed for drought samples. Control and well-watered samples clustered together away from drought samples, except for one drought sample (M48) from *M. sinensis*, which was discarded from down-stream analysis (Fig. 2).

Effects of drought stress on transcriptomes from four Miscanthus **species**

A total of 4,389 of the 67,789 genes (6.4%) in the reference genome were significantly differentially expressed among all species (Fig. 3 and Suppl. Table S4). The highest number of DEGs was observed in *M. x giganteus* (2,353) and the lowest in *M. sinensis* (773). The UpSetR diagram highlights shared DEGs among the four Miscanthus species under drought situation (Fig. 3). Only 67 DEGs were shared by all four species. On the contrary, 3,232 of the 4,389 DEGs (73.3%) were differentially expressed in a single species. On the other hand, only 134 differentially expressed genes were detected in well-watered against control conditions and none of those were shared among all genotypes (Suppl. Fig. S1 and Suppl. Table S5).

Enriched Gene Ontology (go) Terms In Degs

Enrichment analysis of GO terms over-represented among DE genes allowed us to identify the biological processes (BP) and molecular functions (MF) that are regulated in each species during drought. Firstly, we annotated the reference transcriptome with GO and GO-SLIM terms (Suppl. Table S6). The same biological processes were regulated in all the species in the same direction (either up- or down-regulated) and by a similar-enough number of DEGs (Suppl. Tables S7 and S8). This is also evidenced by the similar shape sizes (number of genes), colours (red for up-regulation and blue for down-regulation) and intensities (darker for lower *p*-values) in Fig. 4.

Among the GO terms with greater enrichment among DEGs, (i.e. with lower *p*-value), downregulated differentially expressed genes were significantly involved in protein modification and kinase activity, cell receptor signalling and ion binding; while upregulated differentially expressed genes were significantly involved in sucrose and starch metabolism, redox, and water and glycerol homeostasis and channel activity (Fig. 4). DE genes in these functional categories were functional annotated (Suppl. Table S9) and relevant functions were highlighted in the next result sections.

A similar analysis on the enriched GO slim terms among DEGs during drought highlighted broader GO terms in the similar functions (Suppl. Figure 2); phosphatase activity was consistently upregulated across all species, oxidoreductase activity were significantly down regulated in all species, cellular protein modification process and cellular amino acid metabolic process were significantly down regulated across all species, and response to stress and aging showed consistent upregulation across all species.

Candidate genes involved in starch and sucrose synthesis and degradation

We observed a cluster of three related GO terms (“sucrose metabolism”, “starch metabolism” and “polysaccharide catabolism”), that was up-regulated with strong *p*-values during drought stress and contained a similar number of genes among species (dotted box in Fig. 4). However, sucrose metabolism was not enriched in *M. x giganteus* and none of these GO terms was enriched in *M. sinensis* (most enriched GO terms were not enriched in *M. sinensis*, Fig. 4). The cluster of related GO terms included 53 DEG in total (Suppl. Table S10). Thirty-five of these genes could be mapped to reactions in the starch and sucrose KEGG pathways (Suppl. Figure 3).

Twelve genes were involved in the direct degradation of starch to maltose (3.2.1.1, 3.2.1.2, 3.2.1.68). Ten genes were homologous to BAM1 (Suppl. Table S10; 3.2.1.2) and highly up-regulated with 3.4–8.9 fold-change expressions (FC), six of them were common among the species. Involved in the same process, AMY3 (T282800; 3.2.1.1) had a low 1-1.3 fold-change expression (FC), and ISA3 (4G215400; 3.2.1.68) was weakly up-regulated among the species (0.2-1 FC). On the other hand, two DE glycogen phosphorylase genes were involved in the first step of the degradation of starch in glucose (2.4.1.1), but only one (1G063200) was up-regulated in *M. x giganteus* (3.1 FC) and less so in the triploid hybrid (1.6 FC) and *M. sacchariflorus* (0.7 FC). The related SEX1 gene (18G152900; 2.7.9.4) showed a very similar expression pattern; more strongly up-regulated in *M. x giganteus* (2.4 FC) than in the triploid hybrid (1.3 FC) and *M. sacchariflorus* (0.8 FC).

Concerning starch biosynthesis, waxy gene GBSS-I (19G002300), which synthetises amylose -a starch precursor-, showed very high up-regulation in all *M. sacchariflorus* (11.9 FC), *M. x giganteus* (8.8 FC) and the hybrid 3n (9.3 FC). Two genes involved in the ADP-glucose to starch synthesis, SS3 (T393000; 2.4.1.21) and BE1 (5G197100; 2.4.1.21) were moderately up-regulated in the three species (1.4–2.3 FC and 0.51–1.8 FC, respectively).

SUS3 (1G358800; 2.4.1.13) and two genes encoding SPS1F (16G229500 and 17G242300; 2.4.1.14), which are involved in the last steps of sucrose synthesis, were up-regulated in all species. SUS3 fold-change expression was 1.8–2.6 FC, while SPS1F was 0.52–1.3 FC.

Five cellulose synthase genes involved in secondary cell wall biosynthesis (CESA4 and IRX1/3, Suppl. Table S10; 2.4.1.12) were strongly up-regulated in *M. x giganteus* (5.2–10.4 FC), two were also strongly up-regulated in the triploid hybrids (4.7 and 9.2 FC), but none was in the other species. One glycosyl hydrolase 9B5 (GH9B5) involved in cellulose degradation (3G236400; 3.2.1.4) was up-regulated in *M. sacchariflorus* and the hybrid 3n (2 and 1.7 FC, respectively), but highly up-regulated in *M. x giganteus* (3.7 FC).

Candidate Genesa Involved In Water Homeostasis And Channelling

We observed a cluster of five related GO terms (“cellular water homeostasis”, “water channel activity”, “water transport”, “glycerol transport”, and “glycerol channel activity”), that was up-regulated with strong *p*-values during

drought stress and contained a similar number of genes among species (dotted box in Fig. 4). Within these GO terms in any species, there were thirteen genes in total, twelve of them were aquaporins, and one (18G085200) was homologous to the LRR kinase EREC1/TE1 (“Transpiration efficiency 1”; Suppl. Table S11).

Using rice as a reference, three aquaporins were homologous to PIP2-1 (3G107200), PIP2-2 (7G413400), and PIP2-7 (4G263800). PIP2-7 was lowly up-regulated in all species (0.2-1 FC), while PIP2-2 was only up-regulated in *M. x giganteus* (3.6 FC), and PIP2-1 was up-regulated in *M. sacchariflorus* too (1.2–1.6 FC). Four aquaporins were homologous to PIP1; The homologous genes to PIP1-1 (7G437200) and PIP1-3 (7G548500) were clear, and two additional genes (8G232800 and 12G174400) were homologous to other PIP1 proteins. PIP1-3 (7G548500) was strongly up-regulated in *M. sacchariflorus* (5.3 FC), *M. x giganteus* (3.7 FC) and the triploid hybrid (7.3 FC). NIP2 (7G481100) was highly up-regulated in all species (1.84–2.7 FC) and LRR kinase ER1 (EC 2.7.11.1; ERECTA homolog 1) was only DE in *M. x giganteus* with a low up-regulation (0.2 FC). Four aquaporins had not characterised homologous (1G219200, 3G326300, 8G270100, T569700). The uncharacterised Aquaporin 3G326300 was strongly up-regulated in *M. sacchariflorus* (7.7 FC) alone, but was wholly absent in the triploid hybrid. The uncharacterised aquaporin 1G219200 was only up-regulated in *M. x giganteus* (4.7 FC). All thirteen genes were highly up-regulated in *M. x giganteus*, but only half of them were in *M. sacchariflorus* and the triploid hybrid (Suppl. Table S10).

Discussion

Physiological response to abiotic stress

Miscanthus can relieve pressure on food crops by bio-energy crop, particularly in temperate latitudes [25, 26]. Consecutive-years field trials have shown that annual conversion efficiency for harvestable biomass was significantly higher in *M. x giganteus* (30t/ha) than in switchgrass (10t/ha), under low agricultural input [12]. Adoption of *Miscanthus* as feedstock in marginal lands is challenging. The present study focused on understanding the physiological and regulatory responses in *M. sacchariflorus*, *M. sinensis* their natural hybrid *M. x giganteus*, and a new hybrid, when subjected to water deficit and well-watered (waterlogging conditions).

Electrolyte leakage is a hallmark of plant tissue damage index when plant cells are exposed to abiotic stresses such as drought [27]. In the present study, ANOVA for electrolyte leakage (logarithmic value) revealed a significant difference among the six genotypes, in the three treatment conditions (Table 1). Under drought conditions, the least mean electrolyte leakage was recorded for G5 (*M. x giganteus*) (Table 2; Suppl. Figure 1). Limiting electrolyte leakage, under stress conditions, is positively linked with the plant’s capacity to tolerate the stress in the given time [28]. The fact G5 scored the highest biomass (fresh and dry), under drought conditions, indicates its resilience against soil water deficit (Table 2; Suppl. Figure 3). A similar result was also reported earlier [18] where *M. x giganteus* scored the second-highest dry matter per plant, in a pot study, with reduced water supply.

Several studies, both in greenhouse and field conditions, reported that water deficit reduces photosynthetic capacity and hence significant yield loss in *Miscanthus* [17, 20, 21, 29]. In the present study, biomass yield (fresh or dry weight) was significantly reduced, for all genotypes, under drought conditions in line with previous findings (Table 2/Suppl. Figure 3). Despite the biomass loss under drought conditions, the highest biomass yield (fresh and dry weight) was recorded for G5 (*M. x giganteus*). It was interesting that the newly synthesised hybrid G6 (Hybrid3n), between *M. sinensis* and *M. sacchariflorus*, scored the highest mean fresh and dry weight under well-watered conditions, implying its capacity to thrive under waterlogging conditions. A field study *M. lutarioriparius* was the

highest yielding of the different *Miscanthus* species across the different agro-ecological region in China [30], but a genotype representing this species was not included in the present study.

Another physiological response measured in this experiment was relative water content (RWC), at two-time points. According to [31], a reduction of 5% in RWC can lead to by 40 to 50% reduction in photosynthesis. ANOVA among genotypes revealed a significant difference between genotypes and treatment groups ($P < 0.05$) (Table 1). In drought treatment conditions, the highest mean RWC percentage, for both time-points, was recorded for G4 (*M. x giganteus*) (Suppl. Figure 3/Table 2). On the contrary, despite ca. 10% drop in mean RWC content at the second measurement time point in G5, the same species scored the highest mean fresh and dry weight in drought conditions (Table 2). The highest biomass yield in *M. x giganteus*, under drought conditions observed in the present study contrasts with previous reports where this species showed a lower water-use efficiency than its progenitors (*M. sinensis* & *M. sacchariflorus*) [17, 18, 20]. Such disparity in performance could arise due difference in the experimental set up in addition to genetic diversity. G5 (*M. x giganteus*) is the genotype that scored the least electrolyte leakage under drought conditions.

Changes In Transcript Expression Under Water Stress

The differences in gene expression between four *Miscanthus* species were evaluated by comparing transcriptome changes under control, drought and well-watered treatment conditions within each of the species. From a total of 67,789 mapped transcripts, 4,389, 6.4%, were differentially expressed among four *Miscanthus* species in drought conditions (Fig. 3). The highest DEGs in drought conditions were recorded for *M. x giganteus* (2,353 genes), which also showed a lower reduction in biomass yield under drought conditions compared to the control than the other species. We obtained almost half of the DEG in *M. sinensis* than in *M. sacchariflorus*. A transcriptomics study in water deficit conditions in sorghum showed that the number of DEGs from root samples is much larger than those observed in leaf samples [32]. In our study we only analysed leaf tissues.

All four species showed no significant differences in their transcriptome profile when exposed to well-watered conditions (Suppl. Figure 1). PCA also revealed a similar result, since no clear separation was observed between control and well-watered samples (Fig. 2). The phenotypic assay corroborates this result, both fresh and dry weight measurements were equal or higher in well-watered conditions compared to the control for most genotypes (Fig. 1). Although the present study was conducted under greenhouse conditions, the positive performance of *Miscanthus* genotypes in well-watered conditions indicates that *Miscanthus* could perform well in saturated fields.

Functional Categories Associated With Drought Conditions In Miscanthus

Gene ontology (GO) enrichment analysis allowed us to explore the functions related to drought-responsive genes in *Miscanthus*. While most of the genes were differentially expressed in a single species (Fig. 3), the enrichment analysis of GO terms revealed that the same biological processes were regulated in all the species during stress conditions (Fig. 4). No enriched functional category was observed for *M. x giganteus* and Hybrid3n genotypes (both originating from the same parental species) that was not also seen in *M. sacchariflorus*. Most functional categories were not enriched in *M. sinensis* but that is probably a result of sampling, we did not observe many DEG in the first place.

Sucrose and starch synthesis and degradation were up-regulated with strong *p*-values during drought stress in all genotypes. The up-regulation of several enzymes involved in starch degradation seems consistent with the need to speed up the use of energy reservoirs under stress [33]. Starch biosynthesis is tightly correlated with photosynthesis, another process strongly affected by the environment; a major effect of drought is to reduce transpiration through stomatal closure at the whole plant level. We identified 53 DEGs in total, including ten copies of BAM1, which were highly up-regulated with 3.4–8.9 fold-change expressions in all species (but *M. sinensis*, where we obtained much less DEGs). During osmotic stress, starch is degraded in the light by stress-activated BAM1 and AMY3 to release sugar and sugar-derived osmolytes [34, 35]. Abscisic acid controls the activity of BAM1 and AMY3 in leaves under osmotic stress through the AREB/ABF-SnRK2 kinase-signaling pathway [34]. We also observed a strong up-regulation of GBSS-I, which is involved in amylase synthesis [35]. A common trait of many plants affected by drought or salinity stress is the accumulation of osmoprotectants such as proline, glycine betaine, and sugar alcohols [36].

Twelve aquaporins were up-regulated in across *Miscanthus* species and associated with the enrichment of five GO terms associated with water and glycerol transport and homeostasis. Since many aquaporins (AQPs) act as water channels, they play an essential role in plant water and glycerol relations [37, 38]. *Miscanthus* aquaporins were homologous to multiple isoforms of PIP1, PIP2 and NIP2 in rice and *Arabidopsis*. The highest up-regulation were observed for PIP1-3 across species; also two uncharacterised aquaporins in specific genotypes. As observed here, most plasma membrane intrinsic proteins (PIPs) have a higher level of expression than NOD26-like proteins (NIPs) in *Arabidopsis* [39]. The same paper observed variable regulation (up- or down-regulation) of specific aquaporins in drought stress [39]. However, we observed all of them up-regulated in *Miscanthus*. Another study [40] showed co-expression and physical interaction between PIP1 and PIP2 isoforms in heteromers.

“Protein kinase” and “Phosphorylation” GO terms were significantly enriched among down-regulated DEGs across all *Miscanthus* species under drought conditions. A gene expression study on rice also identified a family of phosphatase proteins regulated during water stress conditions [41]. Expression profiling in response to drought in model species *Medicago truncatula* also identified genes related to phosphatase activity enriched when the plants were under water stress conditions [42]. Overexpression of a single type-I inositol polyphosphate 5-phosphatase (INPP5DP) in *Arabidopsis* increased tolerance to drought under greenhouse conditions [43]. Several studies in grasses reported a significant increase in amino acids accumulation when exposed to water deficit stress [44, 45, 46]. A similar downregulation pattern of gene associated with “cellular protein modification process” and “amino acid modification process” was reported for the California endemic oak (*Quercus lobata*) [47].

Oxidation-reduction process was up-regulated across species, but some DEG in this GO term was also down-regulated (Fig. 3). Similarly, transcriptome profiling in wheat and sorghum in drought conditions showed up-regulation of genes involved in oxidation-reduction process [48, 49].

Some of the GO terms were inconsistently enrichment across *Miscanthus* species. “RNA binding”, “translation”, “ribosome genesis” and “structural ribosome” were related and significantly enriched in *M. sacchariflorus* and *M. x giganteus* but absent from the other two species of *Miscanthus* included in the study (Fig. 3). A previous study in Arabidopsis has shown that different RNA binding proteins play a role in response to drought stress [50].

Upregulation of biosynthetic process BP GO terms was observed among genotypes. Accumulation of secondary metabolites act as antioxidants and minimise adverse effects of water deprivation [51, 52]. Similarly in transcriptomics studies in Arabidopsis under drought stress revealed up-regulation of biosynthetic pathways for phenolic acids and flavonoids [53, 54].

Conclusion

In the present study, a combination of phenotyping under greenhouse conditions and comparative gene expression analysis gave insight into *Miscanthus* species physiological and regulatory response to water stress, either well-watered and droughted conditions. The low number of DEGs in well-watered conditions and higher biomass yield observed in most genotypes supports that *Miscanthus* could be an option in water-saturated arable fields as it was not stressed under very wet conditions. For drought stress a differentiation in phenotypic responses amongst *Miscanthus* species were observed. This study is the first attempting to identify genes playing key roles in response to water stress across and between *M. sinensis, M. sacchariflorus*, and their natural and induced hybrids - *M. x giganteus* and a triploid *M. sinensis x M. sacchariflorus* genotype-. The same biological processes were regulated across species during drought stress despise the identified DEGs were not necessarily the same ones. The noticed critical role of starch metabolism (BAM1, AMY3, ISA3, GBSS-I, SUS3, SPS1F, SS3, BE1, SEX1), cellulose metabolism (CESA4, IRX1/3) and aquaporins (PIP2-1, PIP2-2, PIP2-7, PIP1-1, PIP1-3, ERECT1) in *Miscanthus* species was consistent with functional categories broadly studied and known to be critical during drought stress in model organisms. *Miscanthus* also can offer a relevant model to study the differences in expression resulting from ploidy and heterosis.

Material And Methods

Plant growth conditions and treatment; All genotypes were exposed to three treatment conditions (“control”, “drought” and “well-watered”), in the greenhouse. Each treatment condition; drought, well-water, control was repeated in each of four blocks placed in a green house in a randomized block design. Each genotype was represented by two plants, each in a separate pot. One pot was used for biomass weight measurement (fresh and dry weights), at the end of the experiment, hence untouched. While the other pot was used for taking leaf samples for electrolyte leakage & relative water content (RWC) measurements. All measurements were conducted at the end of the experiment unless stated otherwise.

Plant materials: The physiological experiment was carried out on six *Miscanthus* accessions which were clonally multiplied. Genotype G1: DK-1 (*M. sacchariflorus*), Genotype G2: accession 48 (*M. sinensis*), Genotype 3: (*M. sacchariflorus*), Genotype G4: from Trevor Hodkinson (*M. giganteus*), Genotype G5: ‘Illinois’ (*M x giganteus*) & Genotype G6: S88 (Hybrid3n), new bred triploid.

Water stress

The plants were sown in 11/08/2013 in a greenhouse in 10 × 10 cm pots to and were left to grow to ~ 60 cm height prior to the start of the treatments. Differential water treatment started on 19/08/2013. “Control” received 100 ml water, pots standing on capillary matting. Drought treated plants received 40 ml water, standing on saucers to avoid uptake of water from capillary matting. Pots within well-watered treatment were kept in trays with a continuous water level of between 5 and 8 cm water. The treatment effects on plants were measured via relative water content of leaves and electrolyte leakage of leaves. At the end of the experiments the plants were harvested above the soil and weighed for the determination of fresh and dried biomass.

Soil moisture

Soil moisture measurement was carried out on one of the pots representing each accession, for each of the four blocks, at random time during the running of the experiment. A total of 17 different measurements were recorded

during the experiment. The mean moisture content/treatment group is shown in Suppl. Fig. S4. Soil moisture was measured with a Theta Kit soil moisture instrument from Delta-T Devices Ltd, Cambridge, UK. Three measurements per pot were averaged to determine the soil moisture. Moisture measurement per pot were averaged to determine the soil moisture per genotype.

Relative water content of leaves (RWC) The top 5 cm of the topmost leaf were cut and weighed in tinfoil for the fresh weight (FW). The leaf was submerged in 20 ml distilled water and left in the refrigerator for 24 h. The turgid weight (TW) was determined by blotting the leaf dry and weighing. The leaf was dried for 48 h at 80°c and weighed again for the dry weight (DW). The RWC was then calculated using the formula (FW-DW) / (TW-DW) x 100 = % RWC. Measurements were taken at two time points: 13.09.2013, four weeks (33 days) and 24.09.2013, six weeks (44 days), after water treatment, respectively. The first time point was chosen when the curves on the soil water measurements started to slope visibly with a high chance of having induced significant stress in the plants and the second time point was chosen at the end of the experiment.

Electrolyte leakage

Two leaves were placed in a 50 ml polypropylene tube filled with distilled water. The tubes were closed, covered in tinfoil, and left for 24 h at room temperature. The conductivity in each tube was measured. Afterwards the tubes were capped and autoclaved. After cooling to room temperature, the conductivity of the solutions was measured again. The percentage of electrolyte leakage was calculated as ratio of conductivity before autoclaving and after, the value after represents 100% leakage. Measurements were taken on 25.09.2013 after 45 days in the experiment.

Biomass

Fresh weight was determined for total plant above the soil. Samples for fresh and dry biomass were taken on 25.09.2013. Dry weight was determined after drying the fresh biomass for 48 h at 80 °C.

Statistical analysis

Factors in the phenotypic analyses included block, treatment and time. For analysis of variance (ANOVA) between treatment groups, logarithmic values were used. Where measurements of a response were made at a number of time points, these were included in the analysis as repeated measures and the correlations were modelled using a covariance structure in the Mixed procedure in SAS [47]. Where appropriate, baseline measurements were used as covariates. Tukey adjustments for multiplicity were used for means comparisons and residuals were checked to ensure that the assumptions of the analyses were met.

RNA-Sequencing

Comparative transcriptomics was performed among four *Miscanthus* species, under three treatment conditions (“control”, “drought”, “well-watered”). Four of the six phenotyped *Miscanthus* genotypes did undergo transcriptome sequencing. Leaf samples were taken on 12/09/2013, towards the end of the experiment, and flash-frozen in liquid nitrogen. The selected genotypes were: G1: DK-1 (Msac), G2: accession 48 (Msin), G5: ‘Illinois’ (Mxg) and G6: Hybrid3n, representing each of the three *Miscanthus* species and a newly bred hybrid like *M. x giganteus* (Table 2).

Total RNA was extracted using the RNeasy plant Mini kit from Qiagen according to the manufacturer’s instruction, including an on-column digest of residual genomic DNA. The total RNA was converted into mRNA sequencing libraries using the Illumina TruSeq RNA Sample Preparation Kit (V2) according to the manufacturer’s instructions.

Three biological replicates were taken for each genotype within each treatment group. Therefore, a total of 36 independent libraries were sequenced as 100 bp paired-end reads on an Illumina HiSeq 2000 sequencer. The libraries were multiplexed six times in one sequencing flow cell lane, using six lanes. All raw sequencing data were submitted ArrayExpress (accession number E-MTAB-9354).

RNA-seq reads, pre-processing and alignment

FastQC (v. 11.5) tool, with default parameters, was used to assess raw reads quality, for *Miscanthus* RNAseq each library separately [56]. Thereafter, adapter sequences and low-quality reads were trimmed with Trimmomatic tool (v. 0.38) [57]. All subsequent analyses were performed on reads with a Phred score of + 30 and above and minimal length of 36 bases. Clean reads were aligned to the *M. sinensis* reference genome (*M. sinensis* v7.1 DOE-JGI, http://phytozome.jgi.doe.gov) downloaded from Phytozome with STAR using the “2-pass” mode [58]. The reference was indexed using the *M. sinensis* gene annotation (*M. sinensis* v7.1 DOE-JGI, http://phytozome.jgi.doe.gov) downloaded from Phytozome in GFF3 format. This same gene annotation was functionally annotated with GO terms and enzyme codes with the command-line version of Blast2GO [59] using BLASTX with an E-value of 1e-10 and the NCBI non-redundant (nr) and EBI InterPro databases.

Differential expression and enrichment in gene ontology (GO) terms analysis

The differential expression and enrichment analysis are fully available in an R notebook [60]. Counts were estimated with Stringtie [61]. Differential expression analysis of each treatment against the control group was performed using the DESeq2 R package based on the negative binomial distribution model [62]. Genes with p-value < 0.05 adjusted by Benjamini and Hochberg’s method [63] were considered differentially expressed (DEGs). DEGs shared among four species were visualized with an UpSetR diagram using the R package (v. 1.4) [64]. In order to display the effect of treatment on different species and treatment groups a PCA analysis was carried out with “prcomp” from R and ggplot2 [65].

Enriched GO terms and other categories in each group of differentially expressed genes were identified in R using TOPGO [66] using a Fisher’s test (FDR < 0.05) and the “weight01” algorithm. Using the lists of DE genes and functional annotation as inputs, topGO compared the number of DEGs in each category with the expected number of genes for the whole transcriptome. The “weight01” algorithm resolves the relations between related GO ontology terms at different levels. The relation among GO terms was plotted in R using ggplot [65]. Genes in enriched GO terms were further analyzed in the online Phytomine [67] and Thalemine [68] databases. Genes annotated with enzyme codes were plotted using the online KEGG mapper [69].

Tables and Figures

Table 1: Analysis of variance (REML method), fixed effects are displayed for traits electrolyte leakage, relative water content, fresh weight and dry weight.

Table 2

Estimates of electrolyte leakage, relative water content, fresh biomass, dry biomass and fresh biomass for six genotypes and three treatments (control, drought, well-watered).

Figure 1. Distribution of phenotypic measurements for electrolyte leakage (logarithmic values), relative water content (logarithmic values) at two time points, and fresh and dry biomass weight (in grams, (logarithmic values))

for each genotype across the control, drought and well-watered treatment conditions.

Figure 2. Principal component analysis of the normalised gene counts from RNA-seq libraries generated from four *Miscanthus* species in control, drought and well-watered treatment conditions.

Figure 3. Number of differentially expressed genes (DEGs) shared within and among four *Miscanthus* species under drought conditions.

Figure 4. GO terms (rows) that were significantly enriched ($p < 0.005$) in each *Miscanthus* species (columns) among either up-regulated (top-pointing triangles) or down-regulated (bottom-pointing triangles) differentially expressed genes (DEGs) in drought conditions. The size of a triangle is proportional to the number of DEGs annotated with that GO term. Rows are sorted by descending *p*-value (F-fisher test) and the triangle colour is representative to the obtained p-value, from lower (dark colour) to higher (light colour). Yellow ($p > 0.05$) and white ($p > 0.1$) triangles were not significantly enriched.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data

The RNA-seq data have been assigned ArrayExpress accession E-MTAB-9354. The R code used in the analysis is deposited in Zenodo (http://doi.org/10.5281/zenodo.3950495) and Github (https://joseja.github.io/miscanthus_drought_rnaseq/).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MK & SB designed the study. MK & SB collected phenotypic data. GM & AT analysed the phenotypic data. SB & MK prepared the samples for sequencing. JDV, AT, and SB analysed the transcriptome data. JDV, AT, JF and SB prepared the manuscript. All authors contributed to the final version of the manuscript and approved it.

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Figures

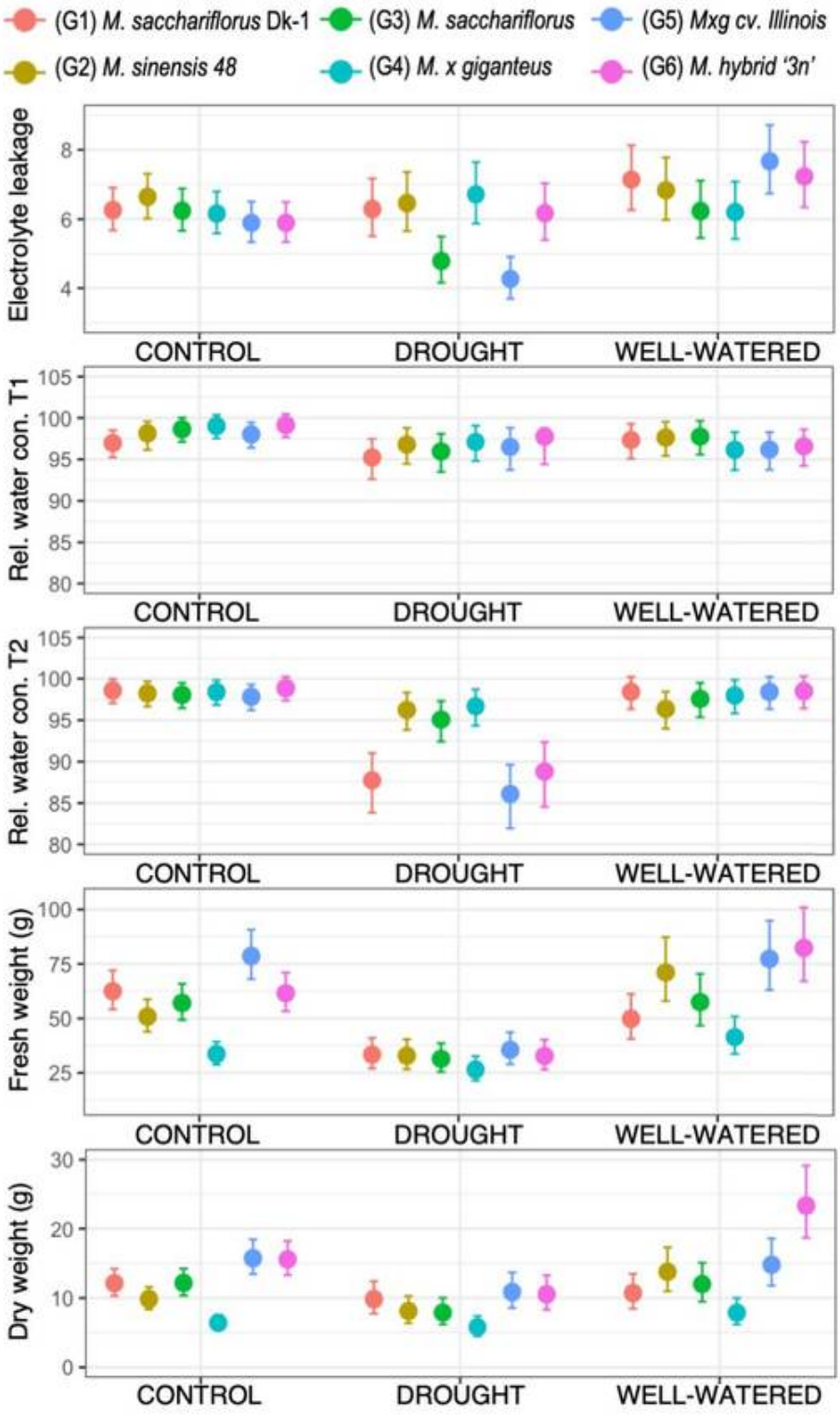

(G1) M. sacchariflorus Dk-1
(G3) M. sacchariflorus
(G5) Mxg cv. Illinois
(G2) M. sinensis 48
(G4) M. x giganteus
(G6) M. hybrid '3n'
Electrolyte leakage
Rel. water con. T1
Rel. water con. T2
Fresh weight (g)
Dry weight (g)
CONTROL
DROUGHT
WELL-WATERED

Figure 1

Distribution of phenotypic measurements for electrolyte leakage (logarithmic values), relative water content (logarithmic values) at two time points, and fresh and dry biomass weight (in grams, (logarithmic values)) for each genotype across the control, drought and well-watered treatment conditions.

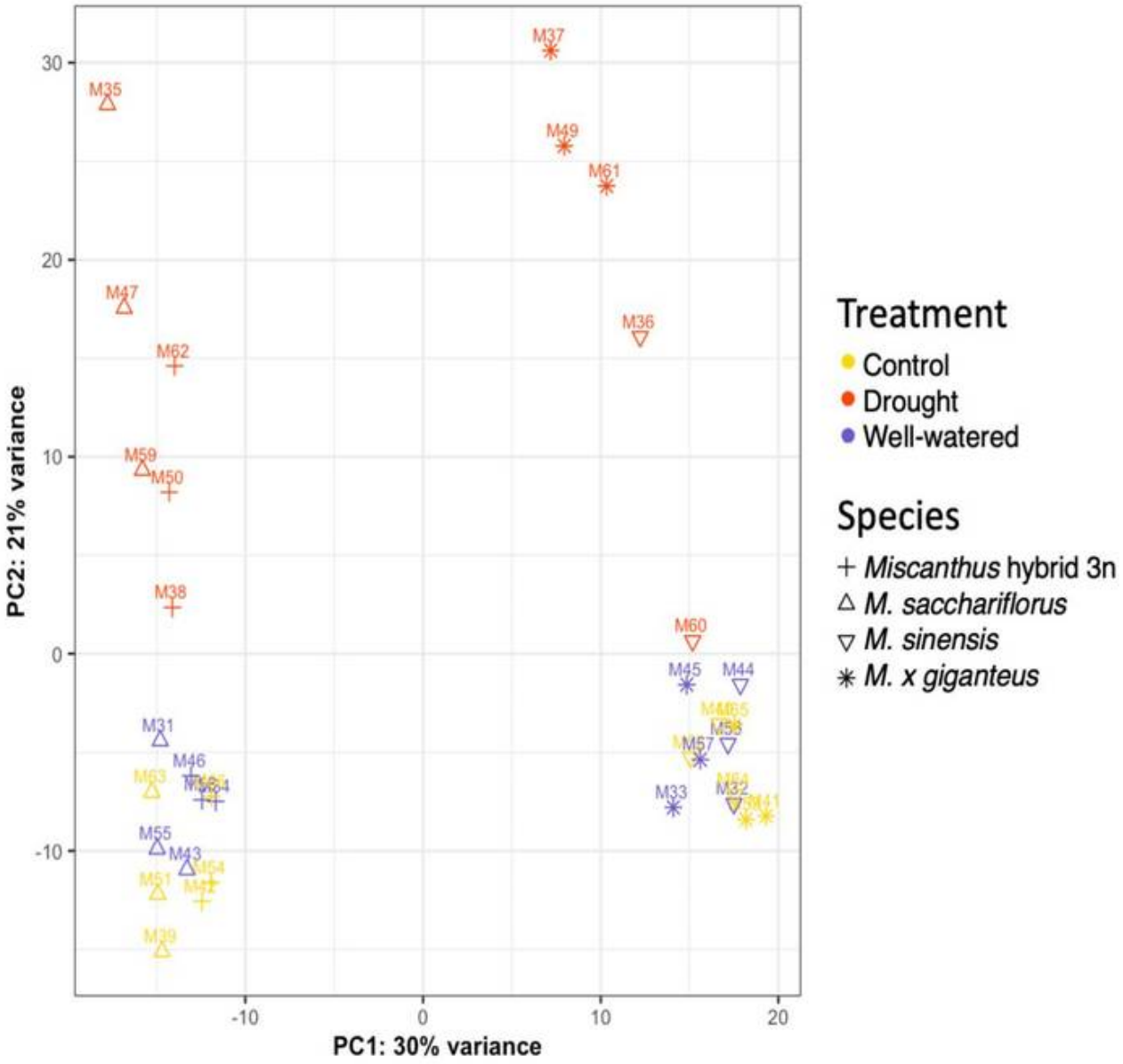

PC2: 21% variance
PC1: 30% variance
30
20
10
0
-10
-10
0
10
20
M37
M35
M49
M61
M47
M36
M62
M59
M50
M38
M60
M45
M44
M31
M46
M57
M33
M32
M55
M43
M54
M51
M39
Treatment
Control
Drought
Well-watered
Species
+ Miscanthus hybrid 3n
△ M. sacchariflorus
▽ M. sinensis
✳ M. x giganteus

Figure 2

Principal component analysis of the normalised gene counts from RNA-seq libraries generated from four Miscanthus species in control, drought and well-watered treatment conditions.

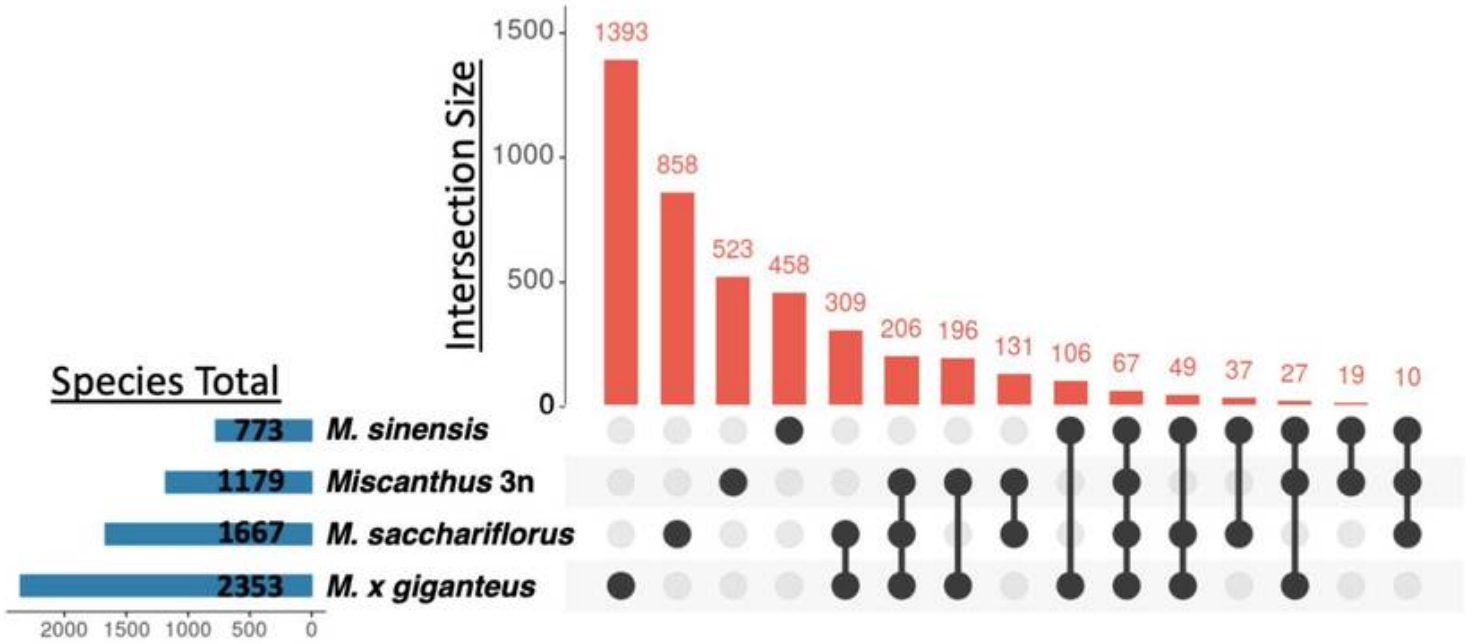
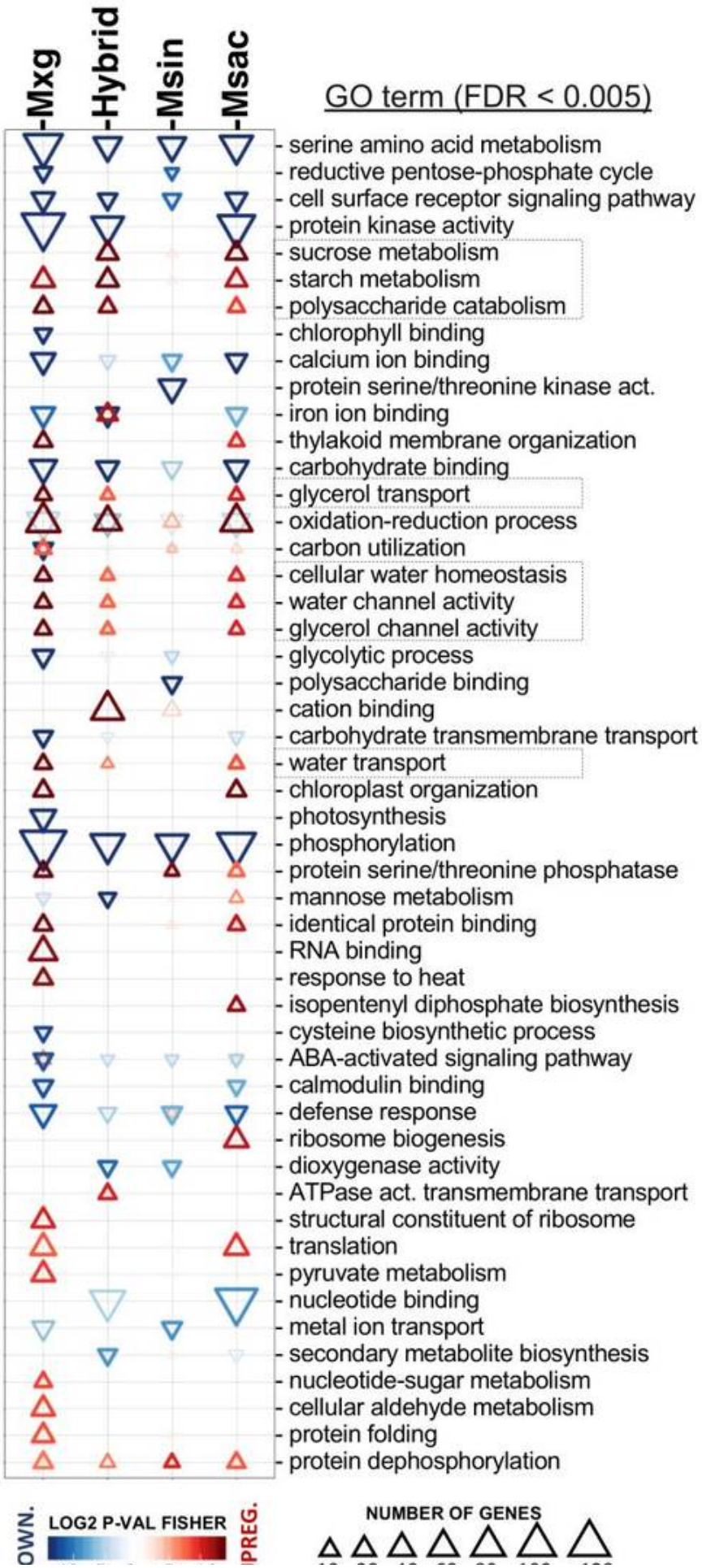

Intersection Size
1500
1000
500
0
1393
858
523
458
309
206
196
131
106
67
49
37
27
19
10
Species Total
773
M. sinensis
1179
Miscanthus 3n
1667
M. sacchariflorus
2353
M. x giganteus
2000
1500
1000
500
0

Figure 3

Number of differentially expressed genes (DEGs) shared within and among four Miscanthus species under drought conditions.


-Mxg
-Hybrid
-Msin
-Msac
GO term (FDR < 0.005)
- serine amino acid metabolism
- reductive pentose-phosphate cycle
- cell surface receptor signaling pathway
- protein kinase activity
- sucrose metabolism
- starch metabolism
- polysaccharide catabolism
- chlorophyll binding
- calcium ion binding
- protein serine/threonine kinase act.
- iron ion binding
- thylakoid membrane organization
- carbohydrate binding
- glycerol transport
- oxidation-reduction process
- carbon utilization
- cellular water homeostasis
- water channel activity
- glycerol channel activity
- glycolytic process
- polysaccharide binding
- cation binding
- carbohydrate transmembrane transport
- water transport
- chloroplast organization
- photosynthesis
- phosphorylation
- protein serine/threonine phosphatase
- mannose metabolism
- identical protein binding
- RNA binding
- response to heat
- isopentenyl diphosphate biosynthesis
- cysteine biosynthetic process
- ABA-activated signaling pathway
- calmodulin binding
- defense response
- ribosome biogenesis
- dioxygenase activity
- ATPase act. transmembrane transport
- structural constituent of ribosome
- translation
- pyruvate metabolism
- nucleotide binding
- metal ion transport
- secondary metabolite biosynthesis
- nucleotide-sugar metabolism
- cellular aldehyde metabolism
- protein folding
- protein dephosphorylation
DOWN.
LOG2 P-VAL FISHER
UPREG.
NUMBER OF GENES

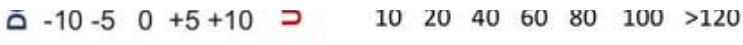


Figure 4

GO terms (rows) that were significantly enriched ($p < 0.005$) in each Miscanthus species (columns) among either up-regulated (top-pointing triangles) or down-regulated (bottom-pointing triangles) differentially expressed genes (DEGs) in drought conditions. The size of a triangle is proportional to the number of DEGs annotated with that GO term. Rows are sorted by descending p-value (F-fisher test) and the triangle colour is representative to the obtained p-value, from lower (dark colour) to higher (light colour). Yellow ($p > 0.05$) and white ($p > 0.1$) triangles were not significantly enriched.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplTableS11.xlsx
- SupplTableS10.xlsx
- SupplTableS9.xlsx
- SupplTableS8.xlsx
- SupplTableS7.xlsx
- SupplTableS6.xlsx
- SupplTableS5.xlsx
- SupplTableS4.xlsx
- SupplTableS3.xlsx
- SupplTableS2.xlsx
- SupplTableS1.xlsx
- deVegaTeshomeetal.SupplementaryFigures.docx