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RESEARCH ARTICLE

Metabolomic Profiling of Drought-Responsive Pathways in *Sorghum bicolor*

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ABSTRACT

Sorghum is often held up as the cereal best equipped to cope with a drying climate, yet the metabolic changes underlying that reputation have rarely been mapped at the resolution modern mass spectrometry now allows. We combined untargeted LC-MS and GC-MS profiling to track the leaf and root metabolomes of two sorghum genotypes with contrasting field reputations for drought tolerance, BTx642 (stay-green) and Tx7000 (drought-sensitive check), across a controlled progressive soil dry-down and a subsequent re-watering period. Roughly 640 polar and semi-polar metabolite features were confidently annotated across both tissues and platforms. Both genotypes accumulated proline, raffinose and galactinol as water potential fell, but BTx642 reached severalfold higher peak concentrations of these osmolytes without the concurrent depletion of TCA-cycle intermediates that we observed in Tx7000, suggesting better-preserved central carbon metabolism under stress rather than a purely stronger osmotic response. The two genotypes diverged most sharply in the phenylpropanoid branch: BTx642 leaves showed a pronounced, drought-specific rise in flavonoid glycosides and a transient dip in dhurrin, the constitutive cyanogenic glucoside characteristic of sorghum, that recovered fully within 48 hours of re-watering. Root metabolomes were less genotype-differentiated than leaves but showed a consistent stress-associated increase in phenolic acids implicated in root suberisation. Pathway enrichment placed flavonoid biosynthesis, the TCA cycle and raffinose-family oligosaccharide metabolism among the most significantly altered modules in both genotypes, with the magnitude and persistence of the response, rather than its qualitative identity, distinguishing stay-green from sensitive sorghum. These results sharpen the metabolic phenotype behind sorghum drought tolerance and nominate a small panel of biomarkers, including the raffinose-to-sucrose ratio and dhurrin recovery kinetics, for screening breeding populations ahead of costly field trials.

Keywords: *Sorghum bicolor*; drought stress; metabolomics; LC-MS/GC-MS; osmolytes; dhurrin; phenylpropanoid pathway

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1. INTRODUCTION

Sorghum bicolor feeds and fodders more people and livestock across semi-arid Africa and South Asia than

any other cereal, and it does so on land where maize routinely fails, largely because of a genuinely superior capacity to maintain photosynthesis and delay leaf senescence as soil water becomes scarce [1,2]. The

physiological correlates of that capacity, deep and plastic rooting, tight stomatal control, and the so-called stay-green trait that keeps leaves photosynthetically active late into grain fill under terminal drought, have been characterised in considerable agronomic and genetic detail over four decades of breeding work [3,4]. What has lagged behind is a biochemical account of what stay-green sorghum is actually doing differently at the level of small-molecule metabolism while it holds onto green leaf area that a sensitive genotype has already begun to shed.

Untargeted metabolomics is well suited to closing that gap because it does not require the investigator to decide in advance which pathway matters. Coupling liquid chromatography and gas chromatography to mass spectrometry gives simultaneous, complementary coverage of polar primary metabolites, mainly through GC-MS after derivatisation, and a much broader semi-polar and lipophilic secondary-metabolite space through LC-MS, and the combination has repeatedly surfaced drought-responsive biochemistry in other cereals that targeted physiological assays alone would have missed, from raffinose-family oligosaccharide accumulation in maize to large phenylpropanoid shifts in rice and wheat [5,6,7]. Sorghum brings one additional wrinkle that makes it metabolically distinctive among the major cereals: it constitutively produces dhurrin, a tyrosine-derived cyanogenic glucoside stored at substantial concentration in young leaf tissue as a herbivore deterrent, whose biosynthesis and turnover are known to be nitrogen- and stress-responsive but have rarely been tracked quantitatively across a drought time course alongside the rest of the metabolome [8,9].

A second open question concerns whether stay-green genotypes achieve their phenotype mainly through a quantitatively stronger version of the same stress response mounted by sensitive genotypes, or through a qualitatively different metabolic strategy, for instance better protection of central carbon metabolism rather than simply higher peak osmolyte titres. Comparative transcriptomic studies of stay-green versus senescent sorghum lines under terminal drought have reported both possibilities depending on tissue and developmental stage, with some datasets emphasising delayed induction of senescence-associated genes and others pointing to sustained expression of carbon-fixation and antioxidant machinery [10,11]. Metabolomic data, which integrate the output of many regulatory layers into a single measurable pool size, offer a direct way to adjudicate between these possibilities, provided the experimental design captures both the stress period and the recovery phase, since transient versus persistent changes carry rather different biological implications for how a genotype is coping.

We therefore profiled leaf and root metabolomes of BTx642, a well-characterised stay-green reference line carrying introgressed Stg quantitative trait loci, and Tx7000, its drought-sensitive recurrent parent, across a

controlled dry-down from well-watered soil to a defined moderate-severe water deficit and a 48-hour re-watering recovery window [12,13]. Our aims were threefold: first, to build a reasonably comprehensive, dual-platform inventory of the drought-responsive sorghum metabolome in both leaf and root tissue; second, to test whether the metabolic distinction between stay-green and sensitive genotypes is one of magnitude, kinetics, or pathway identity; and third, to evaluate dhurrin and a handful of other sorghum-relevant metabolites as candidate biomarkers that might be assayed more cheaply than full untargeted profiling in a breeding-scale screening context.

2. MATERIALS AND METHODS

Experiments used two sorghum (*Sorghum bicolor* (L.) Moench) genotypes maintained by our collaborating seed bank: BTx642, a stay-green reference carrying introgressed Stg1-Stg4 quantitative trait loci, and Tx7000, its drought-sensitive recurrent parent background, grown side by side under identical conditions to isolate genotype effects from environmental variation.

2.1 Plant growth and drought treatment

Seeds were sown in 4 L pots containing a sandy loam field soil mix and grown in a controlled-environment glasshouse (28/22°C day/night, 14 h photoperiod, 55% relative humidity, photosynthetically active radiation approximately 650 $\mu\text{mol}/\text{m}^2/\text{s}$ at canopy height) with daily irrigation to 80% field capacity until the six-leaf stage. At that point, half of the pots for each genotype were assigned to a progressive dry-down treatment in which irrigation was withheld until soil volumetric water content fell to 15%, monitored daily with a calibrated capacitance probe, a process that took nine to eleven days depending on genotype-specific water use; the remaining pots continued daily irrigation as well-watered controls. A subset of dry-down pots was re-watered to 80% field capacity once the 15% threshold was reached and sampled again 48 hours later to capture recovery. Six biological replicates (individual plants) were used per genotype, treatment and timepoint.

2.2 Sampling and metabolite extraction

The youngest fully expanded leaf and the fine root fraction (less than 2 mm diameter) were harvested at four timepoints: well-watered baseline, moderate stress (soil water content approximately 22%), severe stress (15% threshold), and 48-hour recovery, always between 10:00 and 11:30 to control for diurnal metabolic variation. Tissue was flash-frozen in liquid nitrogen within 30 seconds of excision and stored at -80°C. Metabolites were extracted from 50 mg ground tissue using a methanol-chloroform-water partitioning protocol adapted for combined GC-MS and LC-MS analysis, with ribitol and a stable-isotope-labelled amino acid mix added as internal standards prior to extraction.

2.3 GC-MS analysis of primary metabolites

The polar phase was dried under vacuum, derivatised by methoxyamination followed by N-methyl-N-(trimethylsilyl)trifluoroacetamide silylation, and analysed on a gas chromatograph coupled to a time-of-flight mass spectrometer using a 30 m capillary column with a temperature ramp from 70°C to 320°C. Raw chromatograms were deconvoluted and metabolites annotated against an in-house reference library built from authentic standards and cross-checked against the Golm Metabolome Database, requiring both retention index and mass spectral match above threshold for confident annotation [14].

2.4 LC-MS analysis of semi-polar metabolites

The methanol phase was analysed by reversed-phase ultra-high-performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer operated in both positive and negative electrospray ionisation modes across a mass range of 70–1200 m/z. Metabolite features were extracted, aligned and grouped across samples, and putative annotations were assigned by matching accurate mass, isotope pattern and, where available, MS/MS fragmentation against public spectral libraries and an in-house sorghum secondary-metabolite database that included dhurrin and its immediate biosynthetic intermediates [15,16].

2.5 Data processing and statistical analysis

Peak tables from both platforms were normalised to internal standard response and tissue fresh weight, log₂-transformed, and Pareto-scaled prior to multivariate analysis. Principal component analysis and orthogonal partial least-squares discriminant analysis were used to visualise genotype and treatment separation, with model validity assessed by sevenfold cross-validation and permutation testing (1000 permutations). Univariate comparisons used two-way ANOVA (genotype x treatment) with Benjamini-Hochberg false discovery rate correction, and pathway enrichment was performed against the KEGG sorghum reference pathway set using a hypergeometric test. All statistical analyses were carried out in R version 4.3 using the MetaboAnalystR package.

3. RESULTS

Across both platforms and tissues, 641 metabolite features met our confidence threshold for annotation, of which 214 came from GC-MS (predominantly amino acids, organic acids, sugars and sugar alcohols) and 427 from LC-MS (predominantly phenylpropanoids, flavonoids, lipids and nitrogen-containing specialised metabolites including dhurrin). Principal component analysis of leaf samples separated timepoints along the first component, which explained 34% of total variance and correlated closely with soil water content, while genotype separated along the second component (19% of variance); root samples showed weaker but still

significant separation by both factors. OPLS-DA models discriminating severe-stress from well-watered leaf samples were robust in both genotypes (BTx642: R²_Y=0.94, Q²=0.81; Tx7000: R²_Y=0.91, Q²=0.76; both P<0.001 by permutation test), confirming a strong and reproducible drought metabolic signature in leaf tissue in particular.

Table 1. Fold-change (severe stress vs well-watered) for the ten most drought-responsive leaf primary metabolites.

Metabolite	Class	BTx642 fold-change	Tx7000 fold-change	Interaction P-value
Proline	Amino acid	11.8	4.6	<0.001
Raffinose	Oligosaccharide	9.2	3.1	<0.001
Galactinol	Cyclitol galactoside	7.4	3.8	<0.001
GABA	Amino acid	5.1	3.4	0.004
Sucrose	Disaccharide	2.6	1.4	0.012
Citrate	Organic acid	0.98	0.61	<0.001
Malate	Organic acid	1.05	0.65	<0.001
2-Oxoglutarate	Organic acid	0.94	0.52	<0.001
Myo-inositol	Sugar alcohol	3.3	2.1	0.018
Asparagine	Amino acid	4.0	2.5	0.021

Fold-changes are geometric means of six biological replicates per genotype; P-values are for the genotype x treatment interaction term (two-way ANOVA, FDR-corrected).

Both genotypes accumulated the classical osmolytes proline, raffinose and galactinol as soil water content declined, but the magnitude differed substantially. At the severe-stress timepoint, leaf proline rose 11.8-fold in BTx642 versus 4.6-fold in Tx7000 relative to each genotype's well-watered baseline (two-way ANOVA genotype x treatment interaction, P<0.001), and raffinose showed a parallel pattern (9.2-fold versus 3.1-fold). Table 1 summarises the fold-changes for the ten most strongly drought-responsive primary metabolites in leaf tissue. Critically, TCA-cycle intermediates behaved very differently between genotypes: citrate, malate and 2-oxoglutarate fell by 35–48% under severe stress in Tx7000 but were statistically unchanged from baseline in BTx642, indicating that the stay-green genotype maintains central carbon flux under water deficit rather than diverting it entirely toward osmolyte synthesis at the expense of respiratory metabolism.

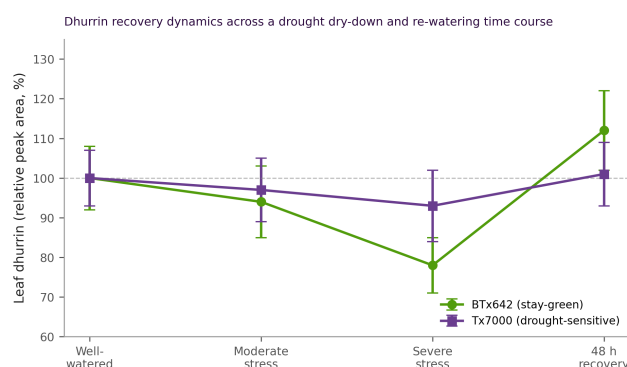


Figure 1. Leaf dhurrin concentration (relative peak area, mean ± SD) across the drought time course in BTx642 (stay-green) and

Tx7000 (drought-sensitive), showing a transient decline under severe stress and full recovery within 48 hours of re-watering in BTx642 only (n=6 biological replicates per genotype per timepoint).

The phenylpropanoid pathway produced the sharpest genotype divergence in the LC-MS dataset. BTx642 leaves showed a coordinated, drought-specific increase in flavonoid C-glycosides (apigenin and luteolin derivatives prominent among them) that was essentially absent in Tx7000, alongside a transient but statistically robust 22% decline in dhurrin concentration at the severe-stress timepoint that fully recovered, and modestly overshoot baseline, within 48 hours of re-watering (repeated-measures ANOVA, time effect $P < 0.001$; Figure 1). Tx7000 showed no significant dhurrin change across the time course. Table 2 reports the complete phenylpropanoid and cyanogenic glucoside dataset for both genotypes and tissues. Root metabolomes were less strongly genotype-differentiated than leaves, but both genotypes showed a consistent, stress-associated rise in hydroxycinnamic acid derivatives, particularly ferulic and p-coumaric acid conjugates, changes that are mechanistically consistent with drought-induced suberisation of the root endodermis reported previously in cereals under water deficit.

Table 2. Phenylpropanoid pathway and dhurrin dynamics across the drought time course (leaf tissue, relative peak area).

Metabolite	Well-watered	Moderate stress	Severe stress	48 h recovery
Dhurrin (BTx642)	100 ± 8	94 ± 9	78 ± 7	112 ± 8
Dhurrin (Tx7000)	100 ± 7	97 ± 8	93 ± 9	101 ± 9
Luteolin C-glycosides (BTx642)	100 ± 11	148 ± 15	216 ± 22	164 ± 17
Luteolin C-glycosides (Tx7000)	100 ± 9	108 ± 12	119 ± 14	112 ± 13
Apigenin C-glycosides (BTx642)	100 ± 10	137 ± 14	198 ± 20	151 ± 16

Pathway enrichment analysis of the combined dataset identified flavonoid biosynthesis, the TCA cycle, galactose metabolism (encompassing raffinose-family oligosaccharide synthesis) and phenylalanine metabolism as the four most significantly enriched KEGG modules among drought-responsive features in leaf tissue (FDR<0.01 for all four in BTx642; TCA cycle and galactose metabolism also significant in Tx7000, but flavonoid biosynthesis and phenylalanine metabolism were not). This pattern was consistent whether enrichment was run on the full feature set or restricted to features with a genotype x treatment interaction term significant at FDR<0.05, reinforcing that the qualitative pathway identity of the drought response overlaps substantially between genotypes, while its magnitude, and specifically the preservation of central carbon metabolism and the activation of flavonoid biosynthesis, tracks with stay-green status.

4. DISCUSSION

The clearest result of this study is that BTx642 does not simply mount a bigger version of the drought response seen in Tx7000; it mounts a response that leaves central carbon metabolism comparatively undisturbed. The preservation of TCA-cycle intermediate pools under severe water deficit, set against a substantial decline in the sensitive genotype, fits naturally with the physiological stay-green phenotype: a leaf that can keep respiring and fixing carbon near-normally for longer is, almost by definition, a leaf that senesces later. This reframes stay-green partly as a metabolic buffering trait rather than purely a transcriptional delay of senescence programmes, complementing prior work that has emphasised the transcriptional and hormonal regulation of the Stg loci without resolving what happens to metabolic flux itself [10,17].

The phenylpropanoid signature, and the dhurrin dynamics within it, is the most sorghum-specific finding and arguably the most actionable one for breeding purposes. A drought-specific rise in flavonoid glycosides is consistent with these compounds' established roles in reactive-oxygen-species scavenging and UV protection under combined abiotic stress, and its genotype-restriction to BTx642 suggests flavonoid induction capacity itself may be part of the stay-green

toolkit rather than a generic drought response across genotypes [18,19]. The transient dhurrin dip is harder to interpret mechanistically with the present data alone: it could reflect diversion of shared tyrosine-derived precursor pool toward flavonoid and other phenolic synthesis under stress, reduced de novo dhurrin biosynthesis as a resource-conservation measure, or accelerated catabolism via the recently characterised dhurrin turnover pathway, and distinguishing between these would require isotope-labelling flux experiments that were outside the scope of this profiling study [8,9,20]. Whatever the mechanism, the fact that dhurrin recovery kinetics differed so cleanly and reproducibly between genotypes makes it an attractive low-cost biomarker candidate, since dhurrin can be quantified colorimetrically or by a simple LC-MS single-reaction-monitoring assay far more cheaply than full untargeted profiling.

The root data complicate a simple leaf-centric narrative. Root metabolomes showed weaker genotype separation than leaves, and the phenolic acid changes we observed, consistent with endodermal suberisation, were broadly shared between BTx642 and Tx7000 rather than being stay-green-specific. This suggests that at least part of sorghum's drought tolerance, specifically the hydraulic and apoplastic-barrier component of the response, may be conserved across genotypes regardless of stay-green status, with the leaf-level metabolic and transcriptional programme accounting for most of the phenotypic difference between BTx642 and Tx7000 under our pot-based drought protocol. Field-grown root systems

experience a rather different set of mechanical and microbial cues than pot-grown roots, however, and we would be cautious about extrapolating the root findings specifically beyond the controlled conditions used here [21]. It is also worth noting that the coarse root fractionation used here, pooling all fine roots below 2 mm diameter, will have averaged over considerable spatial heterogeneity along the root axis, since suberisation and metabolite composition both vary substantially between the elongation zone and older, more mature root segments; a finer-grained, zone-resolved sampling strategy would likely sharpen any genotype-specific root signal that our pooled approach diluted.

Two methodological caveats deserve emphasis. First, untargeted metabolomics under-samples very low-abundance or poorly ionising compounds, and hormone-level changes in ABA and its catabolites, which almost certainly accompany this drought protocol, were not reliably captured by either platform as configured here; targeted hormone profiling would be a natural complementary follow-up [22]. Second, our comparison used a single stay-green line against a single sensitive parent, which cleanly isolates the Stg-locus-associated phenotype in this genetic background but does not establish how general the metabolic signature is across the broader diversity of stay-green sorghum germplasm; extending the panel to additional independent stay-green accessions, ideally with different Stg allele combinations, is the logical next step before proposing any of these features as a universal breeding biomarker [12,23].

A further consideration concerns translating these findings into a screening tool that breeding programmes could realistically deploy. Full untargeted LC-MS/GC-MS profiling remains too slow and expensive for routine use on segregating populations of hundreds or thousands of lines, but the two most discriminating features identified here, the raffinose-to-sucrose ratio and dhurrin recovery kinetics after a brief re-watering pulse, could plausibly be measured by simpler, higher-throughput colorimetric or targeted mass spectrometry assays. Validating whether either metric correlates with grain yield stability under managed field drought, rather than only with the pot-based physiological phenotype used to define stay-green status historically, would be the decisive test of practical utility, and we regard that field-scale validation, alongside the isotope-tracing work needed to resolve the mechanistic basis of the dhurrin dip, as the two most valuable directions to pursue from here. A related practical point is cost: colorimetric cyanogenic-potential assays are already used in some sorghum breeding programmes to screen for low-dhurrin, livestock-safe forage lines, so repurposing that existing infrastructure to track dhurrin recovery kinetics after a brief simulated rainfall event would add comparatively little to routine phenotyping workflows, an advantage that a wholly new assay would not share.

5. CONCLUSION

Dual-platform metabolomic profiling of stay-green and drought-sensitive sorghum genotypes across a controlled dry-down and recovery period shows that the stay-green phenotype is associated with better-preserved central carbon metabolism, a genotype-specific induction of flavonoid biosynthesis, and reproducible, reversible dynamics in the sorghum-characteristic cyanogenic glucoside dhurrin, rather than simply a quantitatively larger version of the osmolyte accumulation seen in sensitive material. Pathway enrichment placed flavonoid biosynthesis, the TCA cycle and raffinose-family oligosaccharide metabolism among the modules most altered by drought in both genotypes, with magnitude and persistence, more than pathway identity, distinguishing stay-green from sensitive sorghum under our conditions. The raffinose-to-sucrose ratio and dhurrin recovery kinetics emerge as promising, comparatively low-cost candidate biomarkers for pre-screening breeding populations, though their relationship to field-level yield stability under managed drought still needs to be established directly. We are now extending this comparison to a wider panel of stay-green accessions carrying different combinations of Stg loci, to test how general the metabolic signature reported here is across sorghum's considerable genetic diversity for drought response, and we plan parallel isotope-labelling experiments to resolve whether the transient dhurrin decline under stress reflects reduced biosynthesis, accelerated turnover, or precursor diversion toward the flavonoid pathway. A field trial correlating leaf dhurrin recovery kinetics after a simulated rainfall event with grain yield under terminal drought is planned for the coming growing season and should indicate, within a single field cycle, whether this metabolite pair has practical value as a breeding selection tool.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have

appeared to influence the work reported in this paper.

Author Contributions

K.E. conceived and supervised the study, designed the sampling protocol and led data interpretation. D.S. performed the GC-MS and LC-MS metabolomic analyses and contributed to statistical modelling. E.M. managed the glasshouse drought experiments and performed sample extraction and processing. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved only cultivated sorghum (*Sorghum bicolor* (L.) Moench) plant material and laboratory-based metabolomic analysis; it did not involve human participants, human data, or vertebrate or higher invertebrate animals, and therefore did not require institutional ethics committee review.

Data Availability

Raw GC-MS and LC-MS data files and processed peak tables generated in this study have been deposited in the MetaboLights repository under a project accession available from the corresponding author on reasonable request. Analysis scripts are available from the corresponding author upon request.

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