Journal of Agronomy and Crop Science

WILEY

Journal of Agronomy and Crop Science

ORIGINAL ARTICLE OPEN ACCESS

Drought Stress

Guard Cell-Specific Metabolic Responses to Drought Stress in Maize

Patrick Pascal Lehr[1] | Alexander Erban[2] | Roman Paul Hartwig[1] | Monika Andrea Wimmer[1] | Joachim Kopka[2] | Christian Zörb[1]

[1]Department Quality of Plant Products 340e, Institute of Crop Science, University of Hohenheim, Stuttgart, Germany | [2]Max-Planck-Institute of Molecular Plant Physiology, Potsdam, Germany

Correspondence: Christian Zörb (christian.zoerb@uni-hohenheim.de)

Received: 22 November 2024 | **Revised:** 27 February 2025 | **Accepted:** 5 March 2025

Funding: This work was supported by State Graduate Scholarship from the Ministry of Science, Research, and Arts Baden-Württemberg, Germany Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) (WI2685/3-2 and ZO118/15-1).

Keywords: amino acids | drought stress | guard cells | maize | metabolome | stomata | stress memory | sugar metabolism

ABSTRACT

Understanding crop responses to drought stress is crucial for securing future agricultural productivity. Guard cells regulate transpiration and thus the yield burden under drought conditions. Therefore, the influence of repeated drought stress on the guard cell metabolome of *Zea mays* L. was investigated to improve our understanding of crop resilience mechanisms. A controlled greenhouse experiment with physiological evaluation and a non-targeted metabolomics approach was used to analyse unprimed and primed guard cells. Primed and unprimed maize plants showed similar overall physiological and metabolic responses to drought, with gas exchange and general metabolic patterns largely unaffected by priming. However, distinct priming effects emerged in specific metabolites. Metabolites of the alanine and aspartate pathway, as well as those of the glycine, serine and threonine pathway were less impacted by drought stress in guard cells than in mesophyll cells, suggesting the emphasis of plants to maintain stable guard cell metabolomes for functional integrity. In contrast, the increase in sugar concentrations in guard cells was similar to that in mesophyll cells, suggesting a pivotal role of sugars in guard cells during drought conditions. New insights into cell type-specific metabolic responses to drought stress will contribute to a better understanding of stress memory in maize. Enhancing guard cell resilience could help optimise water use efficiency for sustainable agricultural production under climate change conditions.

1 | Introduction

Understanding how crops respond to drought stress is crucial in maintaining agricultural productivity amidst climate change. Climate change is expected to heighten the occurrence and severity of drought events in upcoming years. This may result in a larger area of crop-growing land facing drought stress in the future. Maize is significantly impacted by climate change, with drought stress being a key factor for yield losses in maize production. As maize is a crucial crop globally for food, feed, and bioenergy production, the search for innovative methods to alleviate the adverse effects of climate change is highly relevant (Dietz et al. 2021).

Many studies have concentrated on individual stress episodes. Nonetheless, plants in natural habitats or in the field mostly encounter a stress factor such as drought stress multiple times within a vegetation period. Hence, it proves advantageous for the plant to retain a 'stress memory' to enhance its ability to swiftly prepare for subsequent stress responses. This phenomenon can be called 'priming' (Schwachtje et al. 2019). This priming may derive from genotype-specific mechanisms and potentially

---

Summary

- Priming effect influences specific metabolic responses under repeated drought stress.
- Guard cell metabolome remains more stable than mesophyll metabolism during drought.
- Sugars in guard cells follow a distinct regulatory pattern under drought conditions.
- New insights into cell type-specific metabolic responses to drought stress in maize.

synergise with other factors such as short-term acclimations or long-term adaptations at the level of morphology or epigenetics. Another plant strategy is based on a metabolic acclimation that enhances tolerance to stress events (Crisp et al. 2016). Recent studies suggest that stress memory is associated with alterations in transcriptional and metabolic levels (Alves et al. 2020). The presence of a drought memory mechanism grounded in biochemical changes enables plants to efficiently adjust and adapt to varying climatic conditions (Alves et al. 2020). It remains debated whether plants commonly exhibit priming effects or if such effects are rare, with a return to their initial metabolic and physiological state being more prevalent (Wedeking et al. 2018). Investigations of the proteome and transcriptome have been extensively applied to study drought-responsive pathways in maize leaves (Avramova 2015; Schulze et al. 2021; Virlouvet et al. 2018), whereas investigations into metabolic adjustments of maize leaves are still limited.

In earlier studies comparing eight different maize hybrids under drought conditions, KWS STABIL was identified as a suitable genotype for the investigation of drought effects (Kränzlein et al. 2022). This hybrid showed a pronounced increase in electrolyte leakage and a reduction in relative water content under drought conditions, as well as low osmotic adaptability. Despite these characteristics, the reduction in biomass was similar to that of genotypes with an opposite response pattern. Notably, KWS STABIL demonstrated a rapid recovery after drought (Kränzlein et al. 2022). This rapid recovery minimises carryover effects from incomplete recovery in repeated drought cycles, making it particularly appropriate for investigating potential priming effects.

Most previous studies on the effects of drought stress have not focused on special tissues but on the whole leaf. However, in relation to priming, the distribution and spatial organisation of cellular memory in plants is a key open question (Harris et al. 2023; Zörb et al. 2022). It is conceivable that the reaction of cells that experience a specific priming is not uniform. In this context, it is worthwhile exploring the reactions of guard cells, given their crucial function in regulating water consumption. As water availability decreases, a reduction in the turgor of the guard cells occurs, which in turn decreases stomatal opening; this goes along with adaptational guard cell metabolomic changes. Analysis on the level of gene expression showed that P5CS1, a key enzyme for proline biosynthesis, is constitutively higher expressed in guard cells than in mesophyll cells, indicating that the biosynthesis of certain metabolites may be differently regulated in guard cells (Longkumer et al. 2024). When exposed to salt stress, guard cells exhibit distinct metabolomic changes compared to the entire leaf (Franzisky et al. 2021). Considering that the epidermis, mesophyll and vascular tissue may differ in their metabolite response, we here provide novel insight into metabolite regulation in guard cells after recurrent drought. Hence, the impact of recurrent drought stress on the metabolome of *Zea mays* guard cells was analysed by employing a non-targeted metabolomics approach.

2 | Material and Methods

2.1 | Plant Growth and Drought Treatment

The present investigation was conducted in a controlled environment, ensuring high reproducibility and allowing the separation of plant responses to drought and repeated drought from other biotic and abiotic stress factors. *Zea mays* L. hybrid KWS STABIL was selected based on previous results of Kränzlein et al. (2022) and cultivated in a substrate composed of two-thirds sand and one-third arable subsoil, with a maximum water holding capacity corresponding to 15% volumetric soil water content (pF 1.8) (7 L per pot). Cultivation was performed under controlled greenhouse conditions with a 16/8 h day/night cycle, an average maximum day temperature of 34°C, an average minimum night temperature of 23°C, relative humidity ranging from 31% to 64%, and a light intensity of approximately 600 $\mu\text{mol}\,\text{m}^{-2}\,\text{s}^{-1}$. The plants were grown under well-watered conditions (over 10% soil water content) until 18 days after germination. The first drought was initiated on day 18 after germination by withholding water for 4 days until plants showed leaf curling which did not recover after the night. The soil moisture measured herein was set as the target for the second drought stress (5% soil water content). Plants were rewatered from day 22 until day 27 (daily watering) to over 10% soil water content. A second drought was induced by withholding water from day 27 until day 30 when target soil water content was reached. The plants were rewatered to over 10% soil water content for a second recovery cycle until day 33 (daily watering). This experimental design yielded the treatments: consistently watered plants (WW), plants that were fully watered until day 27 and then subjected to the second drought stress (DS2), and plants that were subjected to the first drought stress and then to the second drought stress (primed plants, DS1 + 2) with time point one and time point two for each (Lehr et al. 2024). Mesophyll and guard cell samples (GC) for drought stress 2 timepoint 1 were taken on day 30 and for timepoint 2 on day 33. Nutrients were applied in equal amounts at five points in time, three times before the first drought stress, during the first re-watering and during the second re-watering: 1.26 g nitrogen, 1.24 g phosphate, 0.51 g potassium, 0.68 g magnesium, 2.69 g sulphate and 1.25 g micro nutrient fertiliser (Tenso Cocktail, Yara, Duelmen, Germany) per pot. This experimental setup is essential for understanding how plants respond to drought stress at a mechanistic level.

2.2 | Gas Exchange and Relative Leaf Water Content

The youngest fully expanded leaf before the start of the treatments was used to obtain stomatal conductance and photosynthetic integrity. The measurements were taken every other day (starting on day 18) (Figure 2) using an open gas exchange system (GFS 3000, Heinz Walz GmbH, Effeltrich, Germany) with the measuring cuvette set to 400 ppm CO_2, 15,000 ppm H_2O, 30°C air temperature in cuvette, 1000 $\mu mol\, m^{-2} s^{-1}$ PAR, and gas flow of 750 $\mu mol\, s^{-1}$ (Lehr et al. 2024). Relative leaf water content was measured with the floating rehydration technique (Arndt et al. 2015) using two 1 × 4 cm leaf pieces.

2.3 | Mesophyll and Isolation of Guard Cells

The two youngest fully expanded leaves without a middle vine were cut lengthwise in half. One half was used as mesophyll material, while the other half was used for guard cell isolation. The guard cells were isolated with minor adjustments, following the method previously described by Franzisky et al. (2021). Leaf material for guard cell isolation was blended (ProBland 6 3D, Philips, Amsterdam, Netherlands) in 200 mL deionised H_2O containing crushed ice for 90 s. Subsequently, the tissue was collected and washed with deionised H_2O on a 210 μm pore width nylon mesh (Lehr et al. 2024). The material was subjected to a second blending phase, after which the guard cells were collected on a nylon mesh with a 210 μm pore size, frozen in liquid nitrogen and lyophilised. The guard cells were collected, subjected to liquid nitrogen-based freeze-shock treatment, lyophilised, and stored at −80°C. Guard cell isolation was microscopically checked for effectiveness, and contamination by mesophyll cells was assessed. The isolated guard cells showed a contamination rate of 13.2% (Figure 1).

2.4 | Metabolite Extraction and Metabolite Profiling

Soluble metabolites were extracted using 10 mg of lyophilised and ground mesophyll material or guard cells, according to Erban et al. (2020) with modifications reported by Franzisky et al. (2021). Metabolites were extracted using 360 μL of methanol with U13C6-sorbitol internal standard at 70°C for 15 min. The samples were shaken at 37°C for 5 min after adding 200 μL $CHCl_3$. A liquid phase separation was induced by adding 400 μL of bi-distilled H_2O. Soluble metabolites from guard cells were extracted following the same procedure as for lyophilised ground mesophyll material but using double volumes of extraction solvents (Lehr et al. 2024). Following thorough mixing and centrifugation (20,800 g for 5 min), aliquots of 160 μL of the upper polar phase were dried in a vacuum concentrator overnight at room temperature (Erban et al. 2020). Chemical derivatisation by 40 μL methoxyamination- and subsequently 80 μL trimethylsilylation-reagents was followed by gas chromatography-electron-impact/time-of-flight mass-spectrometry (GasC-EI/TOF-MS)-based metabolite profiling (Erban et al. 2020). This was carried out by

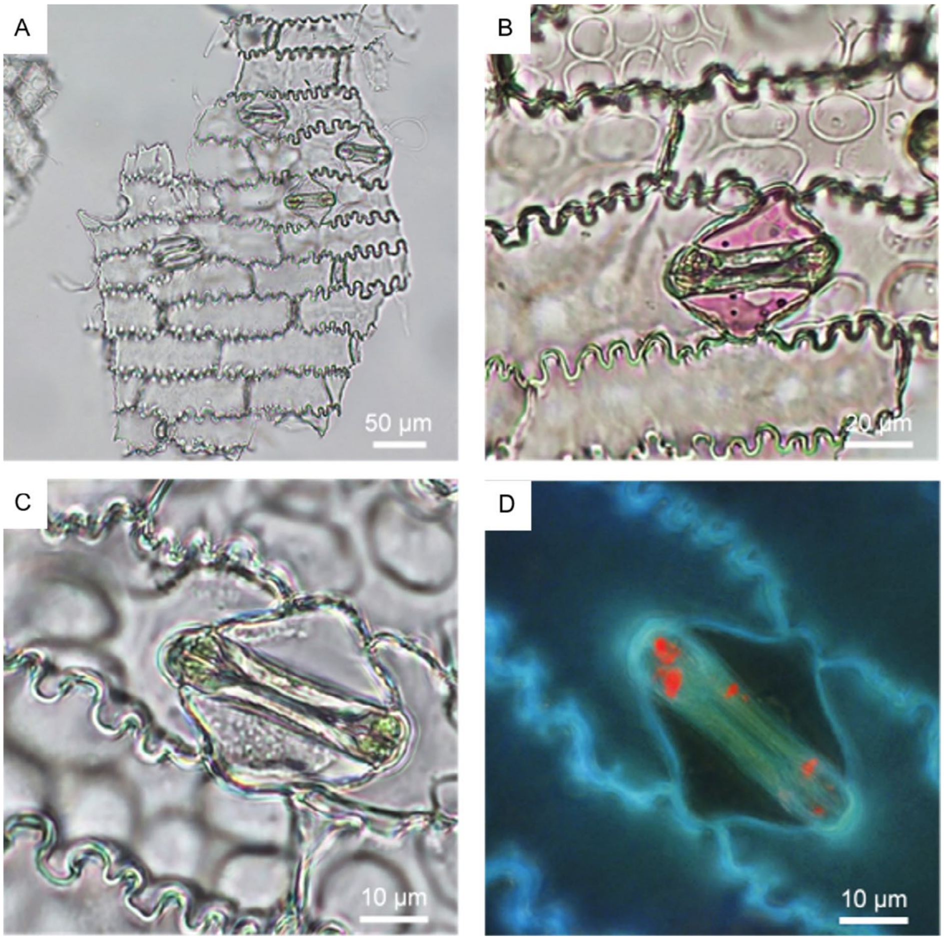

FIGURE 1 | Microscopic images (Axiolab 5, Zeiss, Oberkochen, Germany) of guard cells in bright field (A, B, C), coloured with neutral red (B), and with filter (485/510/575-640) to assess chlorophyll autofluorescence (D).

applying 1 μL of derivatised sample in splitless mode for guard cells and by paired analysis of 1 μL each in splitless and split 1:30 modes for mesophyll material with details reported by Erban et al. (2020). Raw data of amino acids and sugars are according to (Lehr et al. 2024).

2.5 | Metabolite Data Processing

GasC-EI/TOF-MS chromatograms were acquired, visually controlled, baseline-corrected, and exported in the NetCDF file format by using ChromaTOF software (Version 4.22; LECO, St. Joseph, USA). The processing of GasC-EI/TOF-MS data into a standardised numerical data matrix and compound identification were performed by using TagFinder software (Luedemann et al. 2008). Compounds were identified according to standardised guidelines (Erban et al. 2020) by mass spectral and retention time index matching to a reference collection of authenticated standard substances and frequently observed but not yet identified mass spectral tags from the Golm Metabolome Database (GMD) (Hummel et al. 2010; Kopka et al. 2005). Data processing and numerical analysis were carried out according to Franzisky et al. (2021). Metabolite abundancies were normalised to dry weights and the internal standard. For comparison of metabolic changes in response to water deficits, fold changes were calculated in comparison to a well-watered treatment (Lehr et al. 2024).

2.6 | Statistical Analysis

The experimental setup was a randomised complete block design with five biological replicates. Data processing and transformation were conducted using Microsoft Excel (version 1808) (Microsoft, Redmond, USA). The Mack-Skillings test 'PMCMRplus' 'skillingsMackTest' and Dunn's post hock test Benjamini Hochberg with adjusted p-values ($p \leq 0.05$) 'rstatix' 'dunn_test' for multiple comparisons were carried out for results of GasC-EI/TOF-MS data by using 'rstatix' R software (version 4.3.1) (R Foundation for Statistical Computing, Vienna, Austria). A two-factorial ANOVA with both factors timepoint and water treated as fixed effects, followed by Tukey's HSD were carried out for results of photosynthetic integrity and relative leaf water content by using R software (version 4.3.1). Missing values of GasC-EI/TOF-MS data were replaced by half of the minimum value. Data were plotted by using 'ggplot2'. Hierarchical clustering was accomplished by using Spearman Rank Correlation with average linkage clustering. Heatmaps and hierarchical clustering diagrams were drawn by using Multi Experiment Viewer (version 4.9). MetaboAnalyst 5.0 was used to conduct pathway analysis.

3 | Results

3.1 | Similar Photosynthetic Integrity in Primed and Unprimed Plants

The first drought and rewatering cycle until day 27 was used to set priming. First (day 22) and second drought (day 30) were comparable, as indicated by similar soil water content at both drought stresses (Figure 2D). At both drought stresses, *Zea mays* plants showed typical curling of the leaves that was still visible in the morning, as indicated in Figure 2B,C at day 30. The stomatal conductance pattern throughout the experimental period closely mirrored the soil moisture pattern (Figure 2D,E). In comparison to the WW plants at first drought stress, the stomatal conductance of DS1 + 2 plants decreased to 16% and increased to the level of WW plants after 5 days of re-watering (Figure 2E). During the second drought cycle, the stomatal conductance of DS1 + 2 and DS2 plants decreased in parallel as soil water content decreased (Figure 2E). At second drought stress (day 30), the stomatal conductance of DS1 + 2 and DS2 plants was lower than 6% compared to WW plants. After 3 days of rewatering, the stomatal conductance of DS1 + 2 and DS2 plants rebounded, reaching 74% and 83% of the WW plants' stomatal conductivity, respectively. At drought stress two, the relative leaf water content declined significantly from above 83% in WW plants to 44% in DS2 and 52% in DS1 + 2 plants. After 3 days of rewatering, the relative water content returned to the level of WW plants (Figure 2G).

To investigate the integrity of the photosystem, the quantum yield of photosystem II (Phi2), unregulated processes (PhiNO) and non-photochemical quenching (PhiNPQ) light-adapted leaves were used (Figure 2F). The Phi2 accounted for over 50% of the energy under well-watered conditions. Under drought conditions, Phi2 in DS2 and DS1 + 2 accounted for 3% and 7%, respectively, which is significantly lower than in WW plants. Three days after rewatering, Phi2 accounted for over 44% in both DS1 + 2 and DS2 plants. Under drought conditions, the PhiNO decreased significantly from over 34% for WW plants to 27% for DS1 + 2 and DS2 plants. In DS1 + 2 and DS2 plants, PhiNPQ increased significantly from 15% under well-watered conditions to over 65% in drought conditions (Figure 2F). PhiNPQ decreased under 25% for DS1 + 2 and DS2 plants after rewatering.

3.2 | Amino Acid-Containing Pathways Strongly Affected by Drought

A pathway enrichment analysis was performed using all available Kyoto Encyclopedia of Genes and Genomes IDs (KEGG IDs) of the drought-responsive metabolites (Supplemental Figure S1) against the KEGG *Oryza sativa* reference metabolome using MetaboAnalyst 5.0. This analysis evaluates the overrepresentation of study compounds and their pathway impact values derived from metabolic pathway topology analysis. These values indicate the importance of the identified drought-responsive metabolites within their respective metabolic pathways (Zhu and Assmann 2017). Enriched pathways with high impact include aminoacyl-t-RNA biosynthesis, glyoxylate and dicarboxylate metabolism, alanine, aspartate, and glutamate metabolism, galactose metabolism, citrate cycle, glycine, serine and threonine metabolism, carbon fixation and arginine and proline metabolism (Figure S1).

3.3 | Priming Effects for Specific Metabolites

DS1 + 2 and DS2 plants showed a similar pattern according to the metabolic response under drought conditions (Figure 3). The metabolome of DS2 and DS1 + 2 plants returned to a state similar to that of WW plants within 3 days of re-watering after drought stress, especially with regard to amino acids (Figures 5 and 6). Focusing on individual metabolites, it can be seen that in

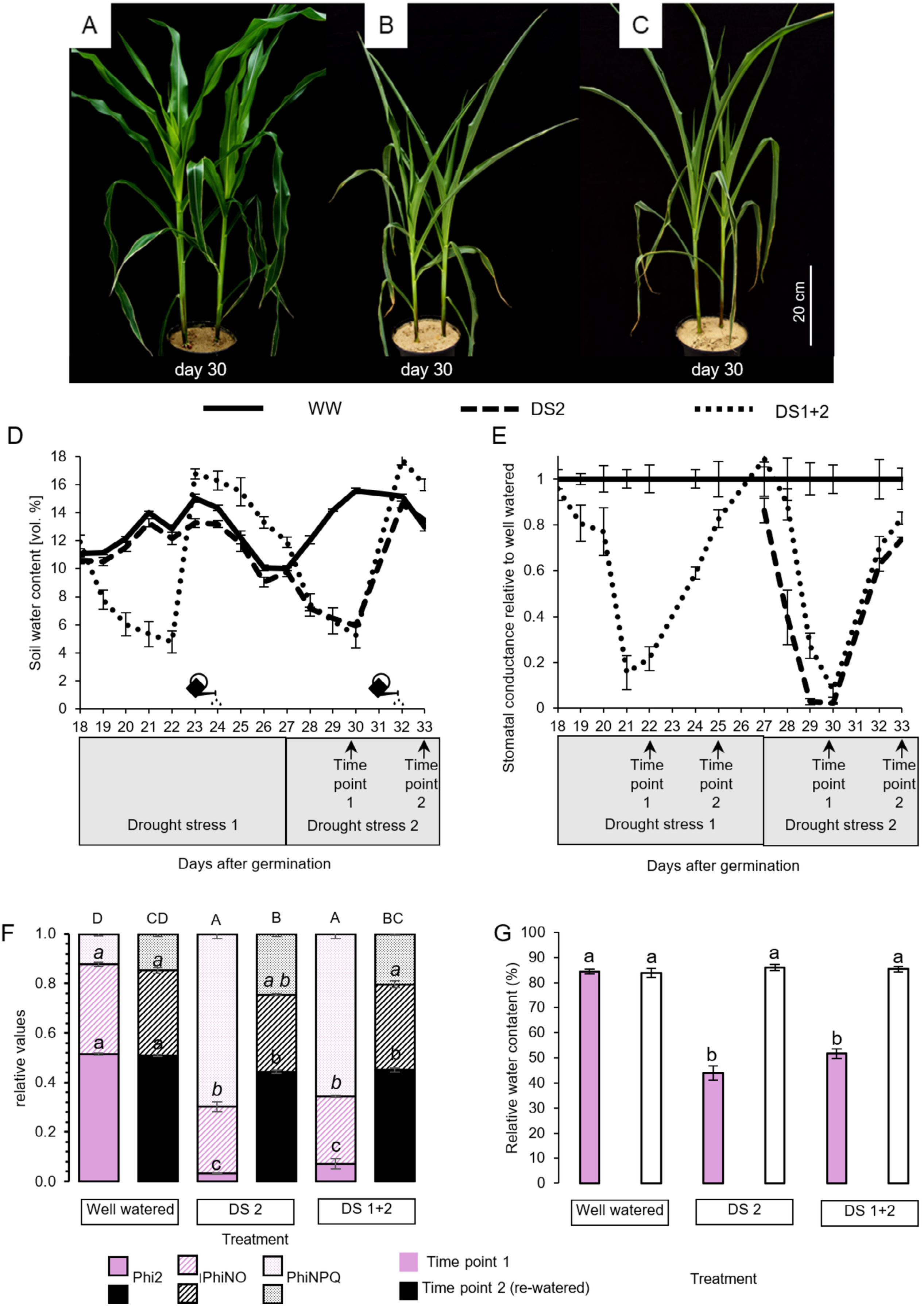

FIGURE 2 | Legend on next page.

FIGURE 2 | Well-watered (WW) (A), drought stressed non-primed (DS2) (B), and drought-stressed primed plants (DS1 + 2) (C), soil water content (D), relative stomatal conductance compared to well-watered plants (E), photosynthetic integrity at drought stress 2 (day 30) (F) and relative leaf water content at drought stress 2 (day 30) (G). Mean values, $n = 5$, ± standard error. Values with the same letters are not significantly different (Tukey's HSD, $p \leq 0.05$). (Figure 1A–G modified after (Lehr et al. 2024)).

some cases priming led to reduced metabolic response. The concentration of proline was 84 times higher in DS2 plants, whereas in DS1 + 2 plants the concentration was over 49 times higher under drought stress when compared to WW plants in mesophyll (Figure 6). GABA increased over 26 times in DS2 plants and 17 times in DS1 + 2 plants under drought conditions when compared to WW plants (Figure 5). The same pattern can be seen for phenylalanine and aspartate, with 16 times and 8 times, respectively, higher concentrations in DS2 plants and 2 times higher concentration in DS1 + 2 plants (Figure 6).

3.4 | Distinct Response Pattern of Guard Cells

Hierarchical clustering showed that the metabolomes of both the mesophyll and the guard cells at drought stress exhibited greater similarity to each other than to the metabolomes of well-watered plants (Figure 4A,B). Furthermore, DS2 and DS1 + 2 plants clustered separately according to their guard cell and mesophyll metabolomes, demonstrating distinct metabolomic profiles within each group. Based on the pathway enrichment analysis (Figure S1) central metabolites of enriched pathways are presented in Figure 3. The concentrations of metabolites connected with the citrate cycle and carbon fixation were decreased in DS2 and DS1 + 2 plants, in mesophyll as well as in guard cells.

Focusing on individual metabolites of influenced pathways, changes in the concentrations of metabolites within glycine, serine and threonine metabolism as well as alanine and aspartate metabolism reveal distinct variations between the mesophyll and guard cells. Metabolites within these pathways exhibited a reduced degree of impact from drought stress in guard cells compared to the mesophyll metabolome. In the guard cells, the increase in serine concentration under drought stress was only one third as high as that observed in mesophyll (Figure 6). A reduced response in guard cells can also be seen in alanine and aspartate, central metabolites of alanine and aspartate metabolism (Figure 6). In mesophyll, alanine and aspartate concentrations increased more than eight times in drought-stressed plants compared to well-watered plants. However, in guard cells, the concentration remained unaffected (Figure 6). In contrast to the minor changes observed in guard cells, a similar increase of glucose and fructose under drought stress in the context of starch and sucrose metabolism was noticed in guard cells compared to the mesophyll (Figures 5 and 6).

4 | Discussion

4.1 | Priming Effects Specific Metabolites Without Altering General Metabolic Acclimation

The comparison of primed (DS1 + 2) and unprimed (DS2) maize plants under drought conditions revealed similarities and specific differences in their responses. While gas exchange was reduced in both plants, primed plants reduced gas exchange slower compared to unprimed plants (Figure 2). This indicates that the overall physiological response to drought stress was similar but with minor modifications (Figure 2). This altered response in gas exchange could not be directly linked to specific metabolites. However, the concentration of several metabolites, including phenylalanine, aspartate and serine, showed a less pronounced increase in primed plants compared to unprimed ones, indicating partial modifications in their drought stress response. Likewise, while the general metabolic responses did not show pronounced differences due to priming, certain metabolites did show distinct changes (Figures 3 and 5). Especially, concentrations of amino acids were influenced by drought treatments, supporting the hypothesis that amino acids may function as osmolytes and substrates for secondary metabolites (Alves et al. 2020; Lehr et al. 2022). A closer examination of individual metabolites uncovered distinct priming effects (Figures 3 and 6). These variations in the responses of primed and unprimed plants may contribute to a more effective acclimation to drought conditions.

The concentration of phenylalanine, a precursor for the phenylpropanoid pathway, was one of the metabolites distinctly influenced by priming (Figure 6). This pathway is integral to the synthesis of compounds like flavonoids and anthocyanins, which enhance antioxidant capacity and protect guard cells from drought-induced oxidative stress (Biała and Jasiński 2018; Abu-Ria et al. 2024). Notably, phenylalanine levels were significantly elevated in unprimed plants under drought stress, whereas the increase was much smaller in primed plants (Figure 6). Considering that photosynthetic integrity, which is a good indicator of oxidative stress, was similar between primed and unprimed plants (Figure 2F), it seems that the highly increased concentration of phenylalanine in unprimed plants did not have an advantage compared to the reduced response in primed plants. This observation suggests that priming leads to a more controlled increase of phenylalanine concentration, potentially enhancing drought resilience without overstimulating this pathway. Priming effects on phenylalanine were more pronounced in mesophyll cells than in guard cells, indicating a cell-type-specific modulation of this metabolite (Figure 6). Despite the overall similarity in metabolic acclimation patterns between primed and unprimed plants, the concentration of specific metabolites such as phenylalanine showed priming effects. These findings show that priming does not necessarily alter entire pathways or plant-wide physiological responses but can induce targeted changes in specific metabolites.

4.2 | High 'Metabolomic Stability' in Guard Cells Under Drought Conditions

Metabolites involved in glycine, serine and threonine metabolism, as well as alanine and aspartate metabolism, were less

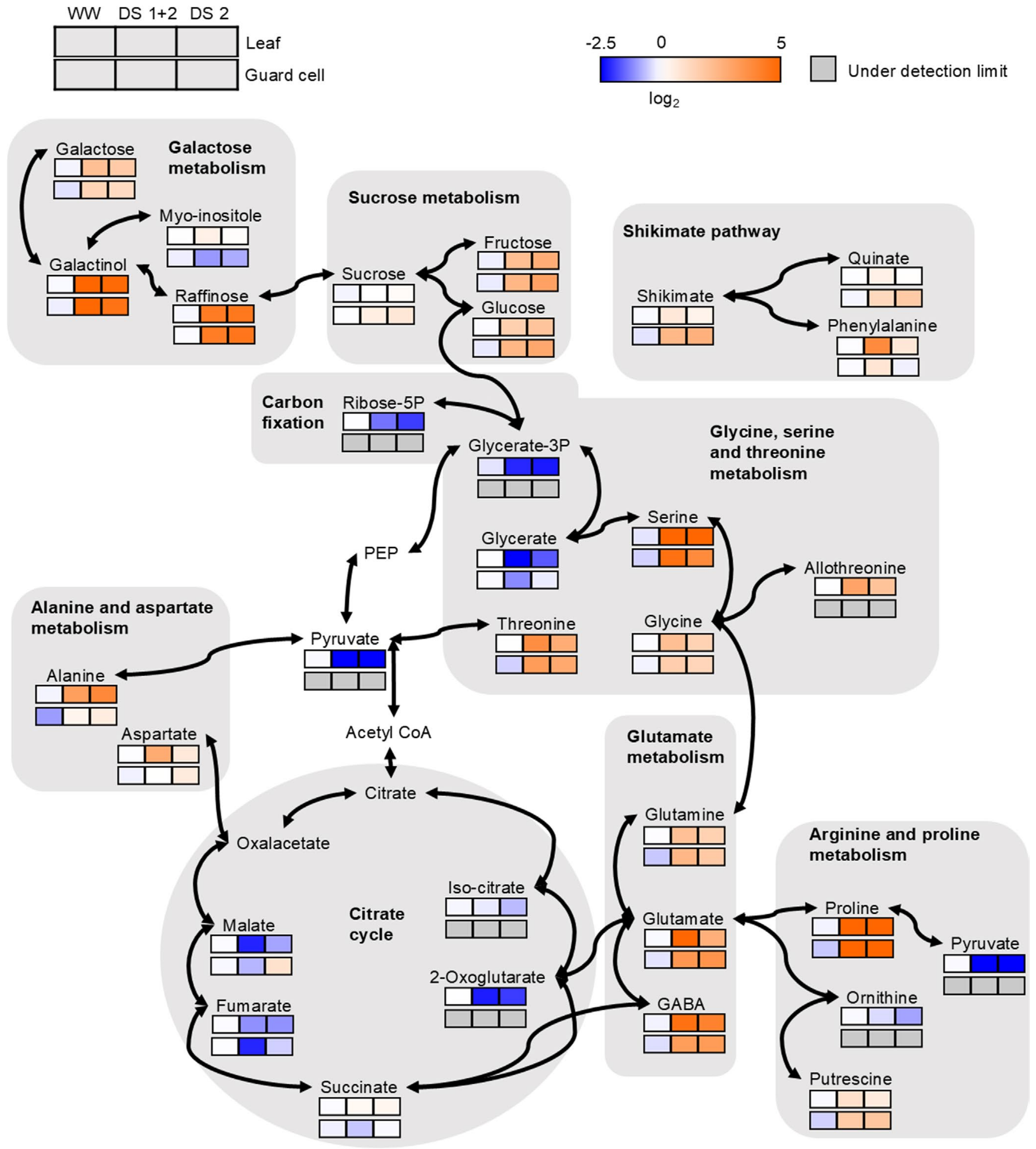

FIGURE 3 | Enriched pathways with high impact (deduced from supplemental Figure S1) and closely connected pathways. A heatmap with $\log_2$ mean values ($n = 5$ and $n = 4$ for DS2 guard cell and DS1 + 2 guard cell) is employed to depict the fold change in metabolite concentrations in unprimed (DS2) and primed plants (DS1 + 2) relative to well-watered (WW) conditions under drought stress (day 30). (raw data of amino acids and sugars according to Lehr et al. 2024).

affected by drought stress in guard cells compared to the mesophyll. Characteristic of this higher metabolic stability of guard cells are alanine and aspartate, the central metabolites of alanine and aspartate metabolism (Figure 6). Alanine is a substrate of the alanine aminotransferase (AlaAT) reaction, which facilitates the interconversion of alanine and pyruvate (Rocha et al. 2010). AlaAT genes were found to be upregulated in *Vaccinium corymbosum* under heat stress, suggesting the involvement of AlaAT in the abiotic stress response (Callwood et al. 2021). Moreover, AlaAT is thought to play a significant role during hypoxic stress conditions, such as those induced by waterlogging or flooding (Rocha et al. 2010). Under drought

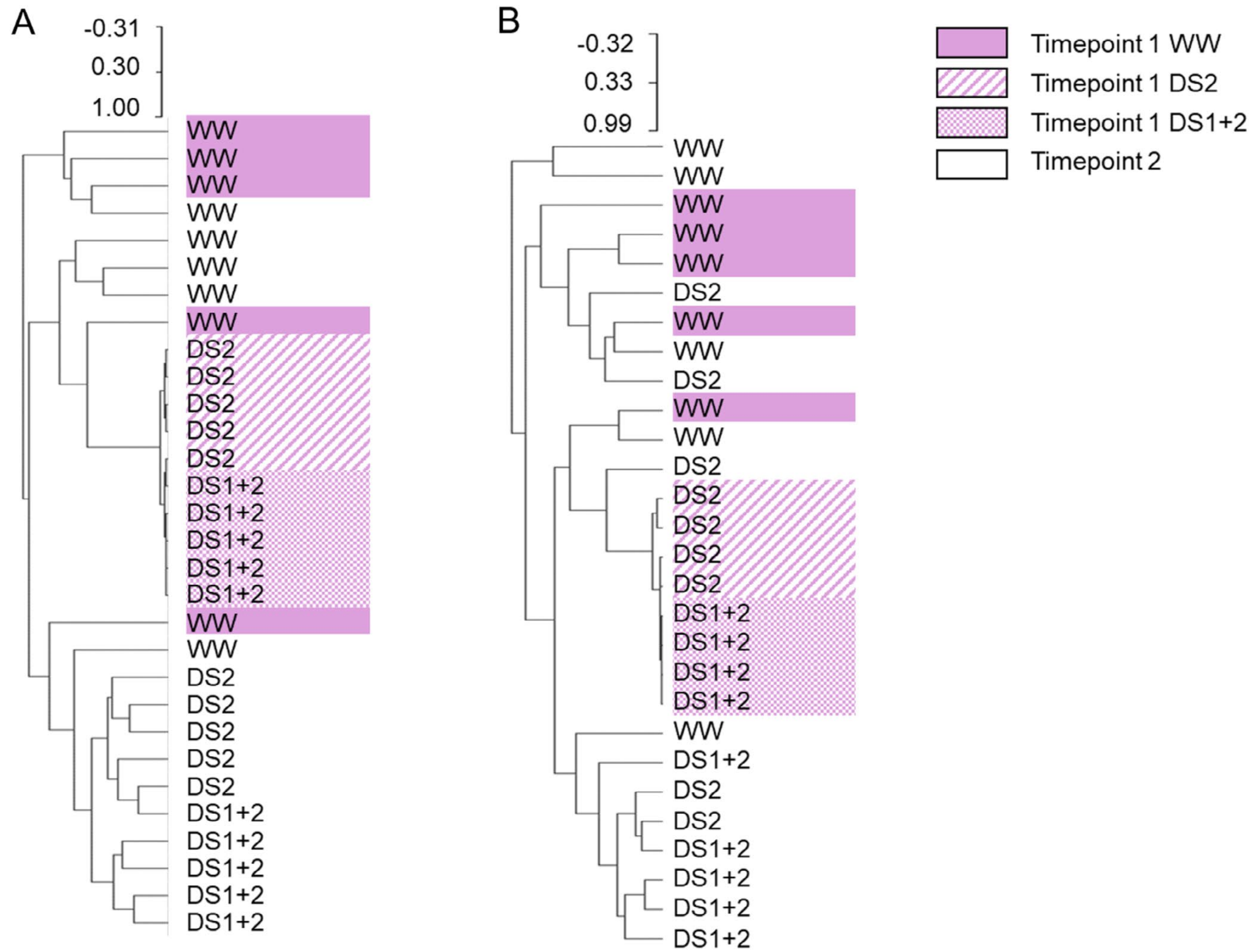

FIGURE 4 | Hierarchical clustering (Spearman Rank Correlation with average linkage clustering) based on all identified metabolites of leaf material (A) and guard cells (GC) (B).

stress, guard cells undergo metabolic reprogramming to meet their energy requirements. Alanine metabolism can contribute to energy supply pathways, such as the tricarboxylic acid (TCA) cycle, through its conversion to pyruvate. This energy is vital for driving ion pumps and channels involved in stomatal closure. Therefore, the unchanged concentration of alanine in guard cells under drought stress may be attributed to its utilisation for energy supply in these cells.

Aspartate concentration in mesophyll cells did not only show a distinct priming effect but was also unaffected in guard cells, whereas the concentration was increased in the mesophyll (Figure 6). Aspartate plays a critical role as a precursor for the synthesis of nucleotides, organic acids and amino acids such as methionine, threonine, lysine and isoleucine (Han et al. 2021). Its function extends to drought response mechanisms, where the aspartic protease ASPG1, containing two aspartate residues, is predominantly expressed in guard cells. This enzyme is involved in ABA-mediated stomatal closure, which reduces water loss and increases drought tolerance (Yao et al. 2012). The present results show a higher stability of aspartate concentrations in guard cells compared to mesophyll cells, even under drought stress (Figure 6). This stability, coupled with the observed priming effects, suggests that aspartate may play a crucial role in adapting plants to recurrent drought events. Specifically, its role in ABA signalling and drought avoidance mechanisms could explain its regulation in primed plants. The observed reduced metabolic acclimation of guard cells compared to mesophyll cells suggests the emphasis of plants in maintaining stable guard cell metabolomes for functional integrity.

4.3 | Robust Energy Supply in Guard Cells

Guard cells typically have fewer chloroplasts compared to mesophyll cells, and their photosynthetic activity is lower (Zhu and Assmann 2017). Additionally, guard cells generally contain more mitochondria than mesophyll cells, which is thought to enable the energy supply required to assist stomatal movement (Zhu and Assmann 2017). It has also been shown that light-dependent metabolic regulation differs between mesophyll and guard cells (Lima et al. 2023). This indicates that it was evolutionarily beneficial to optimise the guard cells mainly for efficient energy supply rather than high photosynthetic efficiency. In relation to energy supply, glucose and fructose are significant energy sources. Although changes in the majority of the analysed metabolites displayed less variation in the guard cells than

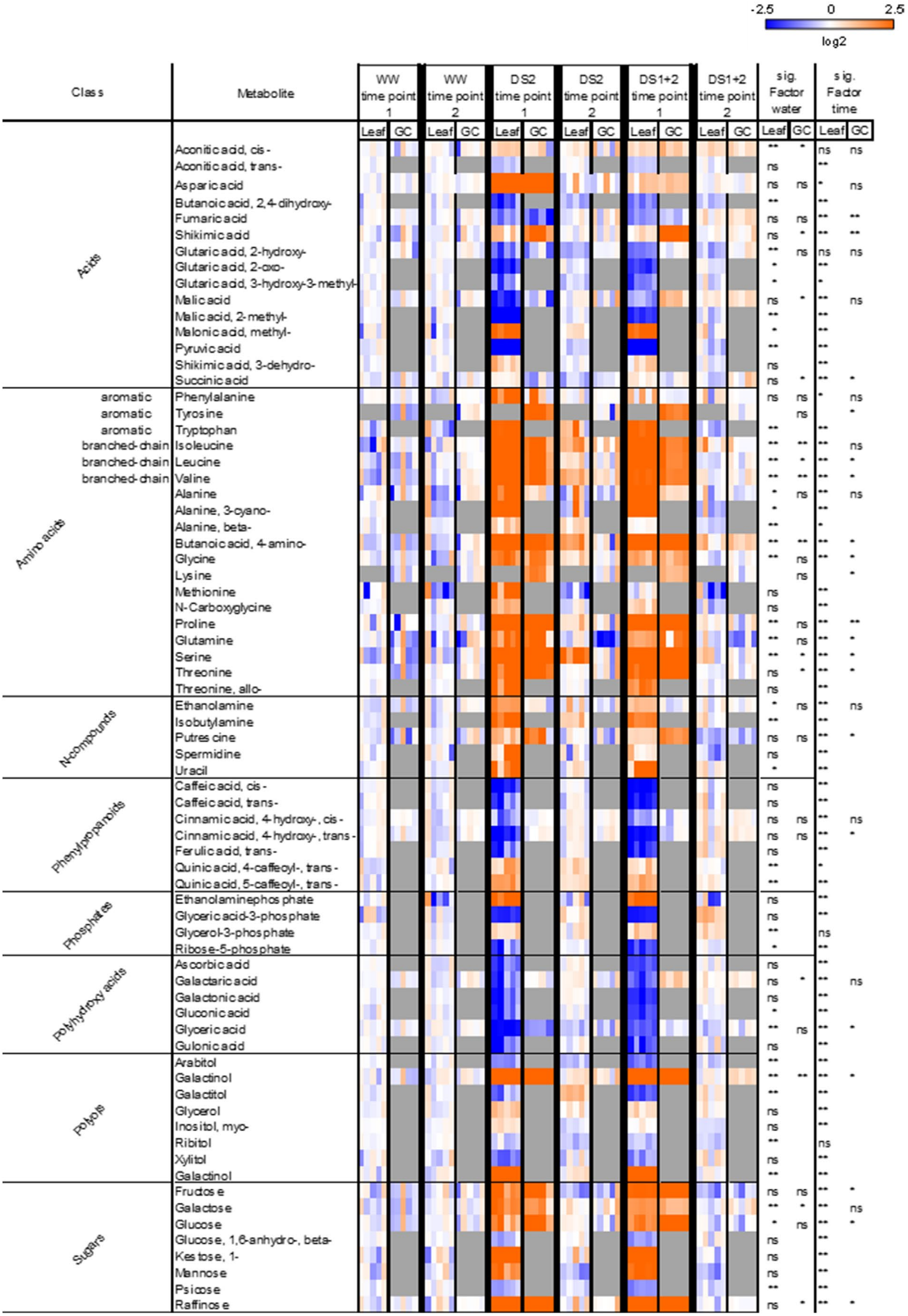

FIGURE 5 | Legend on next page.

FIGURE 5 | Analysis of metabolic changes in leaf material and guard cells (GC) in response to drought stress. Only metabolites significantly influenced by treatments are shown (selection was done by applying Mack-Skillings- test, $p \leq 0.05$). Asterisks indicate the level of significance ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$). The alterations in metabolite concentrations in comparison to well-watered plants, represented as $\log_2$ fold change, are denoted by a colour code for the five biological replicates ($n = 5$ and $n = 4$ for DS2 guard cell and DS1 + 2 guard cell). (raw data of amino acids and sugars according to Lehr et al. 2024).

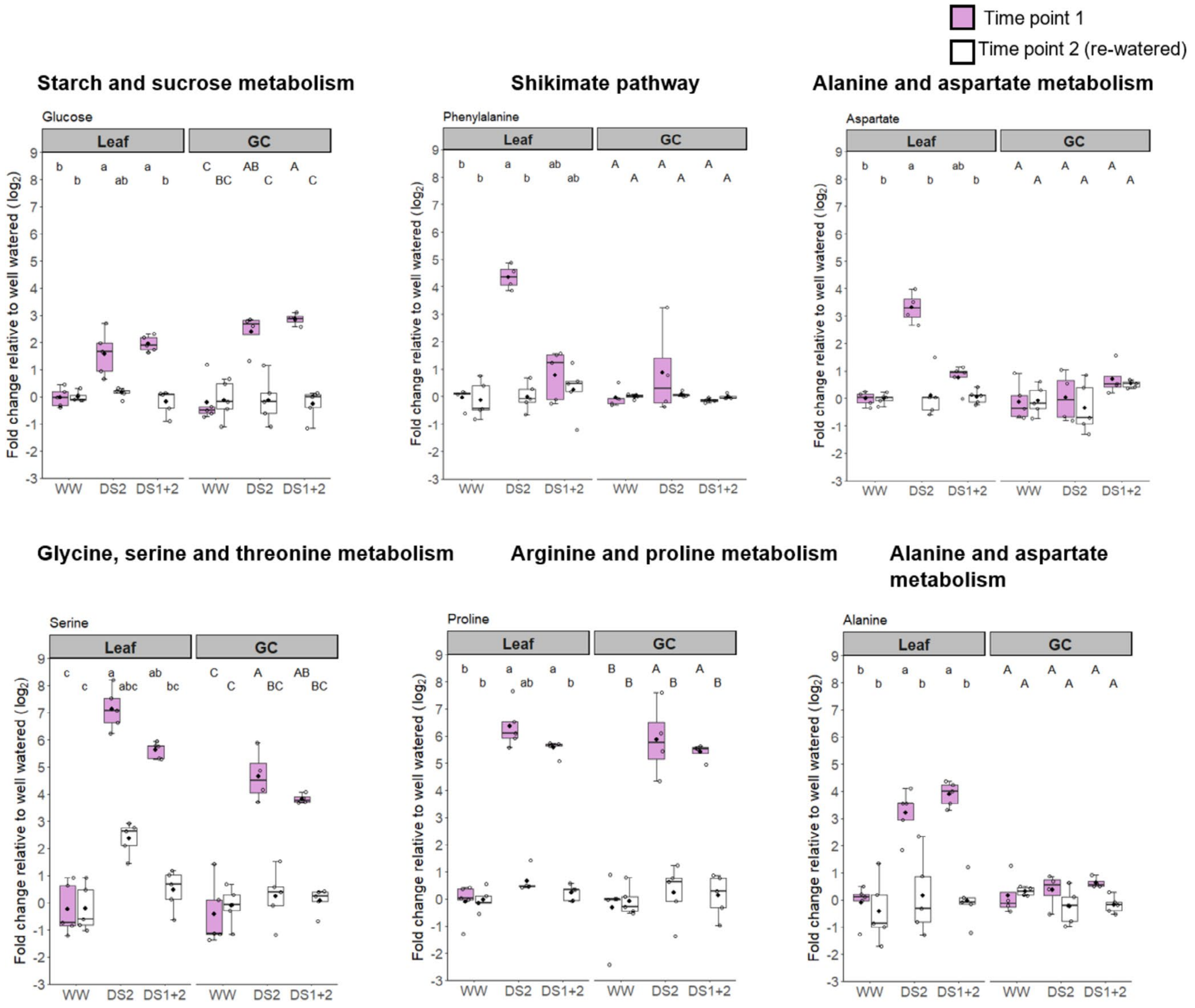

FIGURE 6 | Fold change ($\log_2$) compared to well-watered plants of representative metabolites in leaf material and guard cells (GC) ($n = 5$ and $n = 4$ for DS2 guard cell and DS1 + 2 guard cell). Values with the same letters are not significantly different (Dunn's post hock test, $p \leq 0.05$) (raw data of amino acids and sugars according to Lehr et al. 2024).

in the mesophyll (Figure 5), the opposite was observed for glucose and fructose (Figures 5 and 6). An increase in glucose and fructose under drought stress conditions is a typical response of plants under drought stress conditions to avoid short-term carbon limitation (Sun et al. 2016). A significant increase in glucose and fructose under drought stress with the effort to supply energy to the guard cells enables functionality by maintaining osmotic equilibrium.

A current debate revolves around whether sugars such as glucose, fructose and sucrose do not have an apoplastic osmotic effect on guard cells but instead are sensed within guard cells by hexokinases to stimulate stomatal closure (Kelly et al. 2013; Misra et al. 2015). This might coordinate photosynthesis and sugar levels with transpiration. The phosphorylated hexoses present in guard cells can be metabolised via glycolysis to pyruvate, which can enter the citrate cycle to yield energy (ATP) and various metabolites such as malate, thereby regulating stomatal movement (Misra et al. 2015). Furthermore, the sugars accumulated during the drought phase can help in damage repair and rapid recovery during rewatering (Alves et al. 2020). However, the concentration of sucrose, glucose and fructose increased under drought stress conditions in guard cells (Figure 6), although the source of sucrose in guard cells is not yet clear, as knowledge about the regulation of gluconeogenesis in photosynthetic tissues is limited (Lima et al. 2021). Potentially, sucrose could be acquired through starch degradation in guard cells or an import from mesophyll cells. Nonetheless, the majority of sucrose is believed to be transported from mesophyll cells to guard cells (Misra et al. 2015). This suggests that the energy supply to the guard cells, particularly under drought stress, is of importance for a plant's drought tolerance, achieved through the transport of sucrose into the guard cells.

5 | Conclusion

While gas exchange and general metabolic responses showed a similar pattern between primed and unprimed plants, specific metabolites showed clear priming effects, suggesting targeted metabolic adaptation rather than widespread changes in metabolic pathways. The guard cell metabolome differs notably from the mesophyll during drought, with a comparatively lesser change. Plants prioritise maintaining a stable guard cell metabolome, indicating a guard cell-specific response to drought stress that contributes to their overall drought tolerance. These results suggest that repeated drought may promote a more balanced metabolic acclimation to drought, potentially enhancing crop resilience. Understanding cell type-specific metabolic responses to drought stress will further improve crop productivity under drought conditions, leading to better drought-adapted agricultural production systems.

Author Contributions

C.Z. and J.K.: conceptualization; C.Z., J.K. and A.E.: methodology; A.E., R.P.H. and P.P.L.: formal analysis; P.P.L.: investigation; C.Z. and J.K.: resources; A.E. and P.P.L.: data curation; M.A.W. and A.E.: validation; P.P.L.: writing – original draft; C.Z., J.K., M.A.W., R.P.H., A.E. and P.P.L.: writing – review and editing; R.P.H. and P.P.L.: visualization; C.Z. and J.K.: supervision; C.Z. and P.P.L.: funding acquisition.

Acknowledgements

We thank Ines Fehrle and Dagmar Repper for technical assistance. Graphical abstract created in BioRender. Lehr, P. (2025) (BioRender.com/b45z568). Open Access funding enabled and organized by Projekt DEAL. Declaration of generative AI and AI-assisted technologies in the writing process: During the preparation of this work the authors used ChatGPT 4o to improve language only. After using this tool, the authors reviewed and edited the content as needed, and take full responsibility for the content of the publication.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Abu-Ria, M. E., E. M. Elghareeb, W. M. Shukry, and Others. 2024. "Mitigation of Drought Stress in Maize and Sorghum by Humic Acid: Differential Growth and Physiological Responses." *BMC Plant Biology* 24, no. 1: 514. https://doi.org/10.1186/s12870-024-05184-4.

Alves, R. D. F. B., P. E. Menezes-Silva, L. F. Sousa, et al. 2020. "Evidence of Drought Memory in Dipteryx Alata Indicates Differential Acclimation of Plants to Savanna Conditions." *Scientific Reports* 10, no. 1: 16455. https://doi.org/10.1038/s41598-020-73423-3.

Arndt, S. K., A. Irawan, and G. J. Sanders. 2015. "Apoplastic Water Fraction and Rehydration Techniques Introduce Significant Errors in Measurements of Relative Water Content and Osmotic Potential in Plant Leaves." *Physiologia Plantarum* 155, no. 4: 355–368. https://doi.org/10.1111/ppl.12380.

Avramova, Z. 2015. "Transcriptional 'memory' of a Stress: Transient Chromatin and Memory (Epigenetic) Marks at Stress-Response Genes." *Plant Journal* 83, no. 1: 149–159. https://doi.org/10.1111/tpj.12832.

Biała, W., and M. Jasiński. 2018. "The Phenylpropanoid Case – It Is Transport That Matters." *Frontiers in Plant Science* 9: 1610. https://doi.org/10.3389/fpls.2018.01610.

Callwood, J., K. Melmaiee, K. P. Kulkarni, et al. 2021. "Differential Morpho-Physiological and Transcriptomic Responses to Heat Stress in Two Blueberry Species." *International Journal of Molecular Sciences* 22, no. 5: 2481. https://doi.org/10.3390/ijms22052481.

Crisp, P. A., D. Ganguly, S. R. Eichten, J. O. Borevitz, and B. J. Pogson. 2016. "Reconsidering Plant Memory: Intersections Between Stress Recovery, RNA Turnover, and Epigenetics." *Science Advances* 2, no. 2: e1501340. https://doi.org/10.1126/sciadv.1501340.

Dietz, K. J., C. Zörb, and C. M. Geilfus. 2021. "Drought and Crop Yield." *Plant Biology* 23, no. 6: 881–893. https://doi.org/10.1111/plb.13304.

Erban, A., F. Martinez-Seidel, Y. Rajarathinam, et al. 2020. "Multiplexed Profiling and Data Processing Methods to Identify Temperature-Regulated Primary Metabolites Using Gas Chromatography Coupled to Mass Spectrometry." In *Plant Cold Acclimation: Methods and Protocols*, edited by D. K. Hincha and E. Zuther, 203–239. Springer US. https://doi.org/10.1007/978-1-0716-0660-5_15.

Franzisky, B. L., C. M. Geilfus, M. L. Romo-Pérez, et al. 2021. "Acclimatisation of Guard Cell Metabolism to Long-Term Salinity." *Plant, Cell & Environment* 44, no. 3: 870–884. https://doi.org/10.1111/pce.13964.

Han, M., C. Zhang, P. Suglo, S. Sun, M. Wang, and T. Su. 2021. "L-Aspartate: An Essential Metabolite for Plant Growth and Stress Acclimation." *Molecules* 26, no. 7: 1887. https://doi.org/10.3390/molecules26071887.

Harris, C. J., A. Amtmann, and J. Ton. 2023. "Epigenetic Processes in Plant Stress Priming: Open Questions and New Approaches." *Current Opinion in Plant Biology* 75: 102432. https://doi.org/10.1016/j.pbi.2023.102432.

Hummel, I., F. Pantin, R. Sulpice, et al. 2010. "Arabidopsis Plants Acclimate to Water Deficit at Low Cost Through Changes of Carbon Usage: An Integrated Perspective Using Growth, Metabolite, Enzyme, and Gene Expression Analysis." *Plant Physiology* 154, no. 1: 357–372. https://doi.org/10.1104/pp.110.157008.

Kelly, G., M. Moshelion, R. David-Schwartz, et al. 2013. "Hexokinase Mediates Stomatal Closure." *Plant Journal* 75: 977–988. https://doi.org/10.1111/tpj.12258.

Kopka, J., N. Schauer, S. Krueger, et al. 2005. "GMD@CSB.DB: The Golm Metabolome Database." *Bioinformatics* 21, no. 8: 1635–1638. https://doi.org/10.1093/bioinformatics/bti236.

Kränzlein, M., C.-M. Geilfus, B. L. Franzisky, X. Zhang, M. A. Wimmer, and C. Zörb. 2022. "Physiological Responses of Contrasting Maize (*Zea mays* L.) Hybrids to Repeated Drought." *Journal of Plant Growth Regulation* 41: 2708–2718. https://doi.org/10.1007/s00344-021-10468-2.

Lehr, P. P., A. Erban, R. P. Hartwig, M. A. Wimmer, J. Kopka, and C. Zörb. 2024. "Grapevine and Maize: Two Guard Cell Shaped Strategies to

Cope With Repeated Drought Stress." *Plant Physiology and Biochemistry* 217: 109262. https://doi.org/10.1016/j.plaphy.2024.109262.

Lehr, P. P., E. Hernández-Montes, J. Ludwig-Müller, M. Keller, and C. Zörb. 2022. "Abscisic Acid and Proline Are Not Equivalent Markers for Heat, Drought and Combined Stress in Grapevines." *Australian Journal of Grape and Wine Research* 28, no. 1: 119–130. https://doi.org/10.1111/ajgw.12523.

Lima, V. F., A. Erban, A. Daubermann, et al. 2021. "Establishment of a GC-MS-Based 13C-Positional Isotopomer Approach Suitable for Investigating Metabolic Fluxes in Plant Primary Metabolism." *Plant Journal* 108, no. 4: 1213–1233. https://doi.org/10.1111/tpj.15484.

Lima, V. F., F. B. S. Freire, S. A. Cândido-Sobrinho, et al. 2023. "Unveiling the Dark Side of Guard Cell Metabolism." *Plant Physiology and Biochemistry* 201: 107862. https://doi.org/10.1016/j.plaphy.2023.107862.

Longkumer, T., L. Grillet, H. Y. Chang, et al. 2024. "Insertion of YFP at P5CS1 and AFL1 Shows the Potential, and Potential Complications, of Gene Tagging for Functional Analyses of Stress-Related Proteins." *Plant, Cell & Environment* 47, no. 6: 2011–2026. https://doi.org/10.1111/pce.14861.

Luedemann, A., K. Strassburg, A. Erban, and J. Kopka. 2008. "TagFinder for the Quantitative Analysis of Gas Chromatography-Mass Spectrometry (GC-MS)-Based Metabolite Profiling Experiments." *Bioinformatics* 24, no. 5: 732–737. https://doi.org/10.1093/bioinformatics/btn023.

Misra, B. B., B. R. Acharya, D. Granot, S. M. Assmann, and S. Chen. 2015. "The Guard Cell Metabolome: Functions in Stomatal Movement and Global Food Security." *Frontiers in Plant Science* 6: 334. https://doi.org/10.3389/fpls.2015.00334.

Rocha, M., L. Sodek, F. Licausi, M. W. Hameed, M. C. Dornelas, and J. T. van Dongen. 2010. "Analysis of Alanine Aminotransferase in Various Organs of Soybean (*Glycine max*) and in Dependence of Different Nitrogen Fertilisers During Hypoxic Stress." *Amino Acids* 39, no. 4: 1043–1053. https://doi.org/10.1007/s00726-010-0596-1.

Schulze, W. X., M. Altenbuchinger, M. He, M. Kränzlein, and C. Zörb. 2021. "Proteome Profiling of Repeated Drought Stress Reveals Genotype-Specific Responses and Memory Effects in Maize." *Plant Physiology and Biochemistry* 159: 67–79. https://doi.org/10.1016/j.plaphy.2020.12.009.

Schwachtje, J., S. J. Whitcomb, A. A. Pereira Firmino, E. Zuther, D. K. Hincha, and J. Kopka. 2019. "Induced, Imprinted, and Primed Responses to Changing Environments: Does Metabolism Store and Process Information?" *Frontiers in Plant Science* 10: 106. https://doi.org/10.3389/fpls.2019.00106.

Sun, C., X. Gao, X. Chen, J. Fu, and Y. Zhang. 2016. "Metabolic and Growth Responses of Maize to Successive Drought and Re-Watering Cycles." *Agricultural Water Management* 172: 62–73. https://doi.org/10.1016/j.agwat.2016.04.016.

Virlouvet, L., T. J. Avenson, Q. Du, et al. 2018. "Dehydration Stress Memory: Gene Networks Linked to Physiological Responses During Repeated Stresses of *Zea mays*." *Frontiers in Plant Science* 9: 1058. https://doi.org/10.3389/fpls.2018.01058.

Wedeking, R., M. Maucourt, C. Deborde, et al. 2018. "1H-NMR Metabolomic Profiling Reveals a Distinct Metabolic Recovery Response in Shoots and Roots of Temporarily Drought-Stressed Sugar Beets." *PLoS One* 13, no. 5: e0196102. https://doi.org/10.1371/journal.pone.0196102.

Yao, X., W. Xiong, T. Ye, and Y. Wu. 2012. "Overexpression of the Aspartic Protease ASPG1 Gene Confers Drought Avoidance in Arabidopsis." *Journal of Experimental Botany* 63, no. 7: 2579–2593. https://doi.org/10.1093/jxb/err433.

Zhu, M., and S. M. Assmann. 2017. "Metabolic Signatures in Response to Abscisic Acid (ABA) Treatment in *Brassica napus* Guard Cells Revealed by Metabolomics." *Scientific Reports* 7, no. 1: 12875. https://doi.org/10.1038/s41598-017-13166-w.

Zörb, C., B. L. Franzisky, P. P. Lehr, R. Kosch, M. Altenbuchinger, and C. M. Geilfus. 2022. "Impact of Nutritional Imbalance on Guard Cell Metabolism and Stomata Regulation Under Saline Hyperosmotic Conditions." In *Stomata Regulation and Water Use Efficiency in Plants Under Saline Soil Conditions, Advances in Botanical Research*, edited by S. Shabala, vol. 103, 123–138. Elsevier.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.