Lipidomic remodeling of contrasting maize (Zea mays L.) hybrids under repeated drought

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Abstract

The role of recovery after drought has been proposed to play a more prominent role during the whole drought-adaption process than previously thought. Two maize hybrids with comparable growth but contrasting physiological responses were investigated using physiological, metabolic and lipidomic tools to understand the plants' strategies of lipid remodeling in response to repeated drought stimuli. Profound differences in adaptation between hybrids were discovered during the recovery phase of lipidomic adaptation, which likely gave rise to different degrees of sensitivity to the subsequent drought event. These differences in adaptation are visible in galactolipid metabolism and fatty acid saturation patterns during recovery and may lead to a lipidomic dysregulation in the sensitive maize hybrid. Moreover, the more drought tolerant hybrid displays more changes of metabolite and lipid abundance with higher number of differences within individual lipids, despite of a lower physiological response, while the responses in the sensitive hybrid are higher in magnitude, but lower in significance on the level of individual lipids and metabolites. This study suggests that lipid remodeling during recovery plays a key role in the drought response of plants.

1. Introduction

With drought being a major challenge in modern agriculture, better understanding of drought adaptation and tolerance mechanisms is crucial for breeding of more tolerant maize lines. While plant responses to singular stress events are well understood, responses towards repeated stress events have recently received increased attention, as the ability to recover from stress might have a bigger impact on total adaptability than previously thought (Schulze et al., 2021; Rekowski et al., 2021). The time after the first stimulus is thought to be a crucial phase in which plants may retain metabolic signals of the stress event (retaining imprints) or they return to a phenological pre-stressed state to restart and maximize growth (recovery). The phase in which signals are retained is referred to as stress memory or stress imprint (Walter et al., 2011; Crisp et al., 2016; Wedeking et al., 2018; Hilker & Schmülling, 2019). These different strategies (retaining a memory vs. recovery) are viable under different environmental conditions, if the post-stress environment is characterized by shorter, less severe stress events, the recovery- oriented strategy might be advantageous, while the memory strategy might be better at dealing with longer, more severe upcoming stress periods which are intermitted by longer periods of ambient conditions (Skirycz & Inzé, 2010, Crisp et al., 2016). It has been observed that plants which had experienced a non-lethal initial drought stress are able to survive a subsequent severe drought stress (Crisp et al., 2016). This has been attributed to the formation of a stress

memory after the initial drought treatment. The mechanisms associated with maintaining stress memory require careful regulation as they are likely to be energy intensive, thus resulting in reduced growth and yield (Huot et al, 2014). Crop plants which have been bred for higher yield formation may have lost some of the genetic variation related to stress memory formation (Tanksley & McCouch, 1997). Therefore, in addition to yield formation, plant breeding targets now also include traits for resilience to drought stress (Reynolds et al., 2021).

In the context of drought adaptation, lipids have received much more attention in the recent decade, showing that plant lipids are crucial for energy metabolism, stress signalling (Hou et al., 2016), growth and development (Fujii et al.; 2014; Kobayashi, 2016). The cell membrane is comprised of lipids, which are prone to oxidative processes, that are enhanced under drought stress by the production of reactive oxygen species (ROS) in cellular organs like mitochondria, peroxisomes, and chloroplasts. However, beneficial signaling characteristics of ROS in response to stresses, like the activation of defence- and recovery-related genes are also known (Triantaphylidès and Havaux, 2009; Farmer et al., 2013; Huang et al., 2019). Membranes and cellular structures can be protected from the deleterious effects of ROS by an increased antioxidant capacity, or by counteracting through lipid remodeling, such as modulation of membrane fluidity, accumulation of triacylglycerol (TG) for sequestering released cytotoxic free fatty acids (FFA) and diacylglycerol (DG), leading to the formation of lipid droplets (LD) (Liu et al., 2019; Yu et al., 2021). The major phospholipids in the plasma membrane (PM) are the bilayer lipid phosphatidylcholine (PC) and the nonbilayer lipid phosphatidylethanolamine (PE), as well as phosphatidylinositol (PI) and phosphatidylserine (PS) (Yu et al., 2021). The main lipids of the thylakoid membrane are the galactolipids monogalactosyl-diacylglycerol (MGDG), digalactosyl-diacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and the phospholipid phosphatidylglycerol (PG) (Joyard et al., 1998; Kobayashi, 2016). The different shapes of the headgroups (conic shape of MGDG and cylindric shape of DGDG and SQDG) (Shipley et al., 1973) are responsible for the grana-stacking (Demé et al., 2014) and for the functioning of photosynthesis (Williams, 1998; Wang et al, 2011). Under oxidative stress, the highly unsaturated chains of the galactolipids are oxidized (Ferrari-Iliou et al., 1994), causing a disruption of membrane fluidity and photosynthesis. The plant may adapt by increasing the ratio of DGDG/MGDG (Gigon et al., 2004; Shimojima and Ohta, 2011), such that MGDG is downregulated and mainly converted into DGDG and thus stabilize grana stacking (Gasulla et al., 2013). It has also been discussed that the unsaturated chains of the galactolipids serve as scavengers of singlet oxygen, the primary source of ROS in the plastid (Farmer et al., 2013). The increased saturation of galactolipids under abiotic stress is part of membrane lipid remodeling and has been shown to stabilize membrane fluidity and photosynthesis (Sui et al., 2010). It is known that lipid degradation is one of the first responses to water deficit, as the activity of phospholipases and other lipolytic enzymes increases (Sahsah et al., 1998). Furthermore, the reduced cellular water content contributes to a disruption of membrane fluidity and protein interactions, with the chloroplast membrane being the first target for degradation, leading to premature leaf senescence (Quirino et al.,2000; Guo and Gan, 2005). This premature leaf senescence has been shown to be delayed in genotypes showing an increased DGDG/MGDG ratio and higher lipid unsaturation under drought stress in maize (Chen et al., 2018). Even though lipids clearly occupy a central role in stress adaptation, only few studies investigate changes of the lipid profile under repeated stress or dynamic environments.

In this study, we compared two maize hybrids with contrasting physiological, stress-metabolic and lipidomic responses to repeated drought. We conclude that contrasting lipid remodeling patterns may account for a large portion of the different sensitivities of the maize hybrids to drought stress, which is consistent with differences in ion leakage in response to drought. Moreover, the recovery phase turned out to be the most crucial phase which decides over drought tolerance in the upcoming second drought stress in these contrasting hybrids.

Abbreviations

A	**assimilation rate**
CL	**cardiolipin**
d	**day**

A	**assimilation rate**
DG	**diacylglycerol**
DGDG	**digalactosyldiacylglycerol**
DW	**dry weight**
IL	**ion leakage**
FW	**fresh weight**
Glc	**Glc-sterol lipids (eg. Glc-Sitosterol)**
Gs	**stomatal conductance**
h	**hour**
Hex	**hexosylceramide (sphingolipid)**
IL	**Ion leakage**
K	**refers to maize hybrid 'KWS-stabil'**
L	**refers to maize hybrid 'LG30222'**
LER	**leaf elongation rate**
logFC	**$\log_2$-fold change**
LPC	**lysophosphatidylcholine**
LPE	**lysophosphatidylethanolamine**
MAG	**monoacylglycerol**
MGDG	**monogalactosyldiacylglycerol**
PC	**phosphatidylcholin**
PE	**phosphatidylethanolamine**
PG	**phosphatdidylglycerol**
PI	**phsosphatidylinositol**
PM	**plasmamembrane**
PS	**phosphatitylserine**
ROS	**reactive oxygen species**
SQDG	**sulfoquinovosyldiacylglycerol**
TG	**triacylglycerol**
WHC	**water holding capacity**

2. Materials and Methods

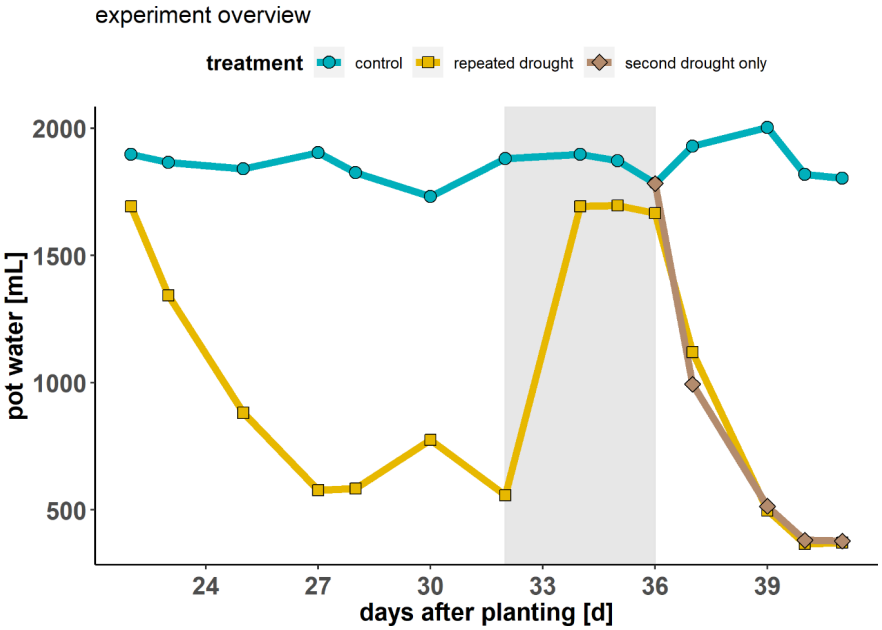

Fig. 1: *Experimental overview: Soil-substrate water content during the phases of the experiment (first drought – recovery – second drought only) for the three treatment groups (control - repeated drought - second drought only). The grey background indicates the recovery phase during rewatering.*

2.1. Plant material and cultivation

Two maize hybrids with contrasting resilience to repeated drought were used based on a previous experiment (Kränzlein et al, 2021). Hybrid L ('LG30222') is considered tolerant to drought and hybrid K ('KWS stabil') is considered more sensitive to drought. 35 seeds per hybrid were soaked for 24 h with aerated 1 mM CaSO4. The day after, on June 26, 2019, the pre-treated seeds were planted in 7 L pots, each containing 4.3 kg of a 1:1 subsoil/ sand-substrate (v/v) mixture. The experiment was conducted in a greenhouse at the University of Hohenheim for a period of 41 d. After each phase of the experiment (see 2.2) on d 31, 35, and 41, five plants per treatment and hybrid were harvested (n = 5). During the experiment, the pots were randomly rearranged once per week to reduce positional effects. Liquid fertilizer (KH2PO4, NH4NO3, MgSO4*7 H2O) was applied once per week, the total amount of nutrients applied was 1.25 g N, 0.5 g K, 0.4 g P, 0.68 g Mg, 0.9 g S.

2.2 Stress treatment

The maximum water holding capacity (WHC) of the substrate-sand mixture was determined at the beginning of the experiment. The WHC of the control conditions was set to 60 %, which corresponds to well-watered conditions based on a pre-experiment using the same hybrids and a similar substrate-sand mixture (Kränzlein et al, 2021). The WHC in the experiment was controlled using weight measurements of the pots at least once per day (Fig 1). The plants were exposed to three treatments: control condition (continuously 60 % WHC), repeated drought treatment (first drought 17 % WHC, second drought ˜ 11 % WHC) and a second drought only treatment (control conditions until the second drought, then ˜12 % WHC). The repeated drought treatment was ˜ 17 % WHC (mild drought) for one week, followed by a 4 d recovery period and a second, terminal drought treatment for 4 d, reaching ˜ 11 % WHC (severe drought).

2.3 Leaf elongation and gas exchange measurements

During the greenhouse experiment, non-destructive measurements including leaf elongation rate (LER) and gas exchange were measured every 1-2 days (see timeline Fig 1). For LER measurements, the length of the

leaf from the surface of the pot to the tip was recorded for all growing leaves. The LER was then expressed in cm h-1 as a mean of the LER of all the elongating leaves per time point. Gas exchange measurements of assimilation rate (A) and stomatal conductance (gs) were conducted on leaves 6 and 7 (counted from the plant base)

throughout the experiment using a LiCl device (ADC BioScientific Ltd., Hoddesdon, England). The same area of leaf 6 and 7 was used for the gas exchange measurements.

2.4 Sampling of plant material for ion leakage, osmotic pressure measurements, metabolomics and lipidomics

For ion leakage measurements, osmolality and metabolomics analyses, fully elongated leaves from leaf 6 and 7 were sampled: leaf disks for ion leakage measurements were taken using a kork borer (0.75 cm diameter), then the middle vein of the remaining leaf was removed, and a subset of the leaf was prepared for osmolality measurements by squeezing the leaf material using a handheld press and freezing the sap immediately at -20°C until the osmolality measurements were taken. The remaining leaf material was frozen in liquid nitrogen for metabolomics analyses. The rest of the plant material that was not used for ion leakage, osmolality and metabolomics analyses was weighted immediately at harvest to record fresh weight (FW) of the shoots. The root material was washed with deionized water, dried in an oven at 50°C for 6 h and the root dry weight (DW) recorded.

2.4.1 Measuring ion leakage and osmolality

For ion leakage (IL) measurements, 10 leaf discs per plant were excised, rinsed for 3 s with MilliQ water, then transferred to a 50 mL centrifugation tube filled with 15 mL of MilliQ water. The tubes were gently shaken for 5 h. The conductivity was measured using a conductometer (WTW LF90; WTW KLE1 cell, Weilheim, Germany). The tubes were frozen overnight and then thawed at room temperature until the solution had equilibrated to room temperature. The final conductivity was recorded afterwards. Total conductivity was then expressed as the ratio of conductivity after 5 h and the total conductivity after thawing. The frozen samples for osmolality measurements were thawed, centrifuged at 11000 g for 10 min, then the osmolality of the supernatant was measured with a vapor-pressure osmometer (VAPRO® Vapor Pressure Osmometer, ELITechGroup, Paris, France) three times per sample.

2.5 Metabolomics measurement

The frozen leaf material was freeze-dried and ground using a mill (Retsch, Germany). Pulverized samples were used for lipidomics and metabolomics. 25 mg of the freeze-dried sample was weighted into a 1.5 mL reaction tube, then 330 µL of a 90/10 (v/v) methanol/water mixture with internal standard (ribitol, 0.05 mg/mL) was added. The mixture was shaken for 15 min at 70°C, then cooled (to RT) and subsequently 230 µL chloroform with standard solution (methylnonadecanoate, 0.25 mg/mL) was added. The mixture was shaken at 37°C for 5 min, afterwards 400 µL MilliQ water was added and then again shaken at RT for 1 min . The samples were centrifuged for 5 min at 14 000 g and an aliquot of 80 µL of the upper polar phase containing the metabolites was taken and dried in a vacuum concentrator. Oximation reagent was 50 mg of 4-(dimethylamino)pyridine dissolved in 10 mL pyridine with subsequently added 400 mg of methoxyamine hydrochloride. Silylation reagent was a mixture of 1 mL N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) and 150 µL retention index solution (containing n-decane, n-hexadecane, n-docosane, n-octacosane und n-tetratriacontane). For derivatization, 40 µL oximation reagent was added to the dried residue, then the solution was shaken for 90 min at 30°C. Subsequently, 80 µL silylation reagent was added, thoroughly mixed for 1 min at 37°C and incubated at 37°C for 30 min. The solution was transferred to a silanized GC-vial and quickly sealed. GC-MS/MS analysis was carried out on an Agilent 7890B gas chromatograph coupled with an Agilent 7000D triple quadrupole mass spectrometer (Agilent, Waldbronn, Germany). The injection volume was 1 µL (splitless). The separation was done on an HP-5MS UI fused silica capillary column (30 m, 0.25 mm I.D., 0.25 mm film thickness) and the injector temperature was set to 250°C, carrier gas was He with a flow rate of 0.6 mL min-1. The temperature program was 70°C (1 min), followed by an increase of 9°C min-1 to 310°C (10 min). The transfer line and source temperature were set to 250°C. The mass selective

detector was operated in scan mode with a mass range of m/z 70–600. The identification of metabolites was done via the NIST database (2017) and standard substances with respect to retention time and mass spectra. The metabolomics procedure is described in Dethloff et al. (2014).

2.6 Lipidomics measurement

Lipids were extracted from the freeze-dried, pulverized leaf material as following the protocol published by Shiva et al. (2018), with minor modifications, reported by Kehelpannala et al. (2020). The freeze-dried maize samples were homogenized by cryomilling (Precellys 24; Bertin Technologies, https://bertin-technologies.com) with 400 µl of 2-propanol containing 0.01% butylated hydroxytoluene (BHT) for two consecutive 45-s intervals, with a 30-s pause in between, at 6100 rev/min and a temperature of -10°C. Next, the samples were incubated at 75°C for 15 min while being gently shaken at 1400 rev/min. Then, they were cooled to room temperature (25°C) and 1.2 ml of a mixture of chloroform (CHCl3)/methanol (MeOH)/water (30/41.5/3.5, v/v/v) was added to each sample. The samples were incubated at 25°C for 24 h with constant gentle shaking. Finally, the solvent was separated, and the sample was dried in a vacuum concentrator. A quality control sample was prepared by combining 10 µl of each sample extract. The dried lipid extracts were re-suspended in 200 µl of butanol (BuOH)/MeOH (1:1) with 10 mM ammonium formate and subjected to LC-MS analysis, as reported by Hu et al. (2008). Extracts were used for untargeted LC-MS lipidomics measurement using the protocol of Yu et al. (2018).

2.7 Statistical analysis

Lipidomic and metabolomic data (with n=4-5 replicates) was normalized by library size and log2-transformed. For statistical analysis and plotting, the program R was used (Version 4.1.1). We stratified the data with respect to hybrid and timepoint and used the "limma" package from the Bioconductor project (Huber et al., 2015) to test lipid-wise for an association with treatment. Pairwise comparisons were corrected for multiple testing using the procedure of Benjamini-Hochberg (Benjamini and Hochberg, 1995). The alpha used for significance of adjusted p-values was alpha < 0.05. The t-statistics from the "limma" function was used to generate a pre-ranked lipid "genes" list, which was used to perform a gene set enrichment analysis using the function "cameraPR" from the "limma" package. The PCA was calculated using normalized and log2-transformed lipid and metabolite data, which were scaled and centered lipid/metabolite-wise prior to PCA. PCA was calculated using the "prcomp" function from the R "stats" package. For the loadings plot, the loadings of the individual lipids were summarized into a single vector pointing towards the center of gravity of the individual lipids within one lipid class to better illustrate the impact of the whole lipid class.

3. Results

3.1 Photosynthesis, leaf elongation, plant weight, ion leakage, osmolality

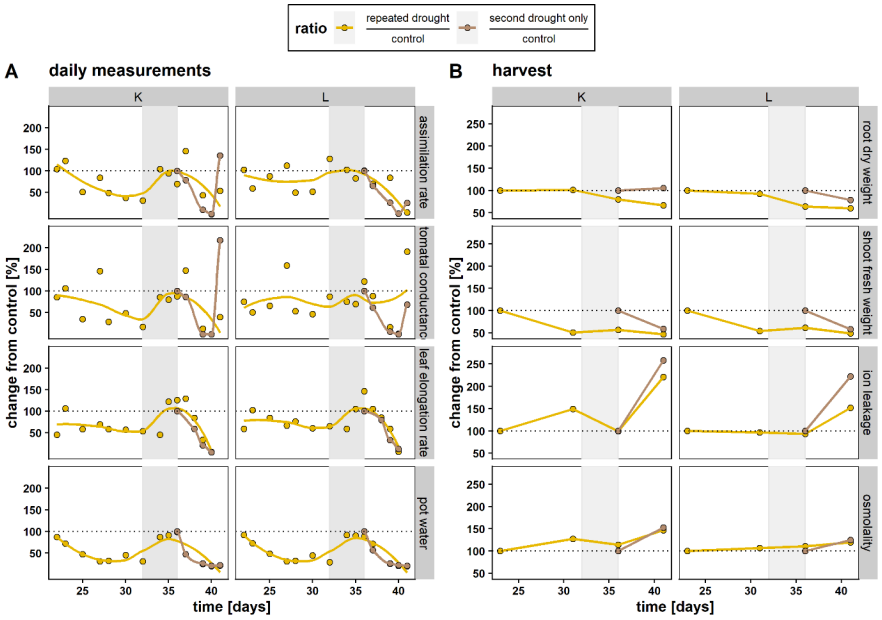

Fig. *2: Responses of maize hybrids K and L to repeated drought treatment (yellow) and the second drought only treatment (brown) relative to well-watered control values in percent (100 %, dotted line). Each datapoint represents the mean of n = 4-5 replicates. Lines were fitted with a Lowess function for curve smoothing. Grey area represents the recovery phase.* ***A,*** *Physiological responses of assimilation rate, stomatal conductance, leaf elongation rate (A, gs and LER respectively) and pot water* ***B,*** *Biomass and stress parameters from destructive sampling. A+B: The first measurement at d 23 of the repeated drought treatment and the first measurement of the second drought only treatment are imputed values to better illustrate changes, because no harvest has taken place before the first drought at d 23 and the second drought only treatment was at control conditions (=100%) after the recovery event.*

Both hybrids exhibited a progressive decrease in gas exchange under drought conditions at single drought and repeated drought, respectively (Fig. 2A). They both showed a return to control conditions during the recovery phase (Fig. 1, grey area). For K, the response of assimilation rate and stomatal conductance (Fig. 2A) was more pronounced (lower in comparison to well-watered control) under the first mild drought, emphasizing higher sensitivity to water deficit. Furthermore, the assimilation rate and stomatal conductance of the repeated drought treatment was less affected at the second drought relative to the second drought-only treatment in both hybrids (Fig. 1 A). The changes in LER during the experiment were relatively similar in both hybrids and treatments. Both hybrids showed a decline in LER under repeated drought and an overcompensation towards the end of the recovery phase. When experiencing only the second drought, both hybrids showed a rapid decline in LER. The root DW and shoot FW responses (Fig. 2B) were similar in both hybrids, while other stress parameters, such as ion leakage and osmolality, revealed the different sensitivities of the hybrids to mild drought. Ion leakage and osmolality in more drought sensitive hybrid K were already elevated under first drought, returned to control levels after recovery, and showed rapid elevation under repeated drought in both drought treatments. On the contrary, in the drought tolerant hybrid L almost no increase in ion leakage and osmolality were detected after the first drought, and only a small increase during the repeated drought (Fig. 1B). Furthermore, hybrid K shows high IL among both drought treatments after second drought (121% and 158% after repeated drought and second drought only, respectively) while hybrid L shows more reduction in IL after repeated drought exposure (52% and 122% after repeated drought and second drought only, respectively). Overall, the results were in agreement with previous data (Kränzlein et al., 2021).

3.2 Overview of metabolic and lipidomic adaptation

For investigating overall adaptation patterns between hybrids across all timepoints and treatments, a PCA was calculated (Fig. 3). The PCA using metabolite and lipidomic data explained 33% of total variance via the first two principal components, PC1 and PC2, while the treatment was mainly separated by the PC1 (right side: severe drought, left side: control, recovery and mild drought). The first drought was mild such that the drought treatments were not separated from control in this projection. However, hybrid L showed a more pronounced adaptation after recovery than K. Second drought treatment led to similar changes in both hybrids mainly on the PC1 axis relative to developmental changes in the well-watered controls. The PC1 component separating the treatments correlated most with L-valine, malic acid, pentanedioic acid, glyceric acid, glycolic acid, shikimic acid, LPC (lyso-phosphatidylcholine) and MGDG (Fig. 3B), which are known stress-responsive metabolites.

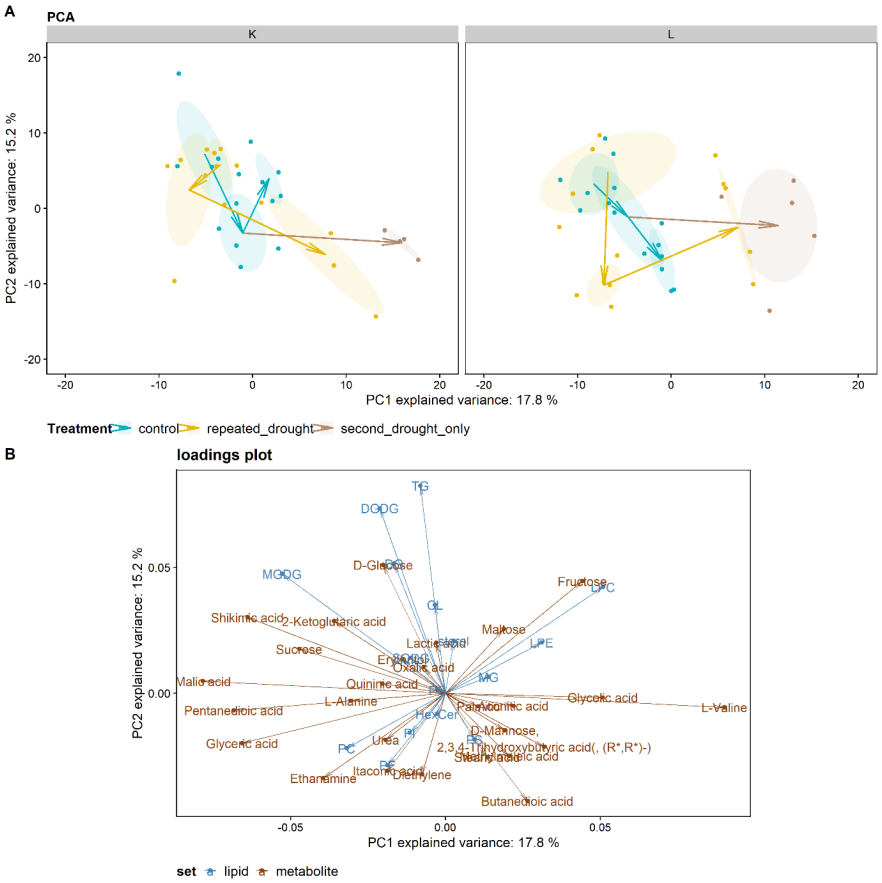

Fig. 3: A, *PCA plot facetted by hybrid. Arrows indicate the progression of time (first drought – recovery – second drought) in each treatment group. Ellipses indicate 95% confidence intervals of the center of gravity of each subgroup. The second drought only treatment stems from the control treatment after recovery;* ***B,*** *plot illustrating the loadings for PCA projection. The arrows which belong to lipid groups point towards the center of gravity of the single lipids within the respective lipid group to better illustrate the impact of the whole lipid group.*

3.3 Metabolite responses

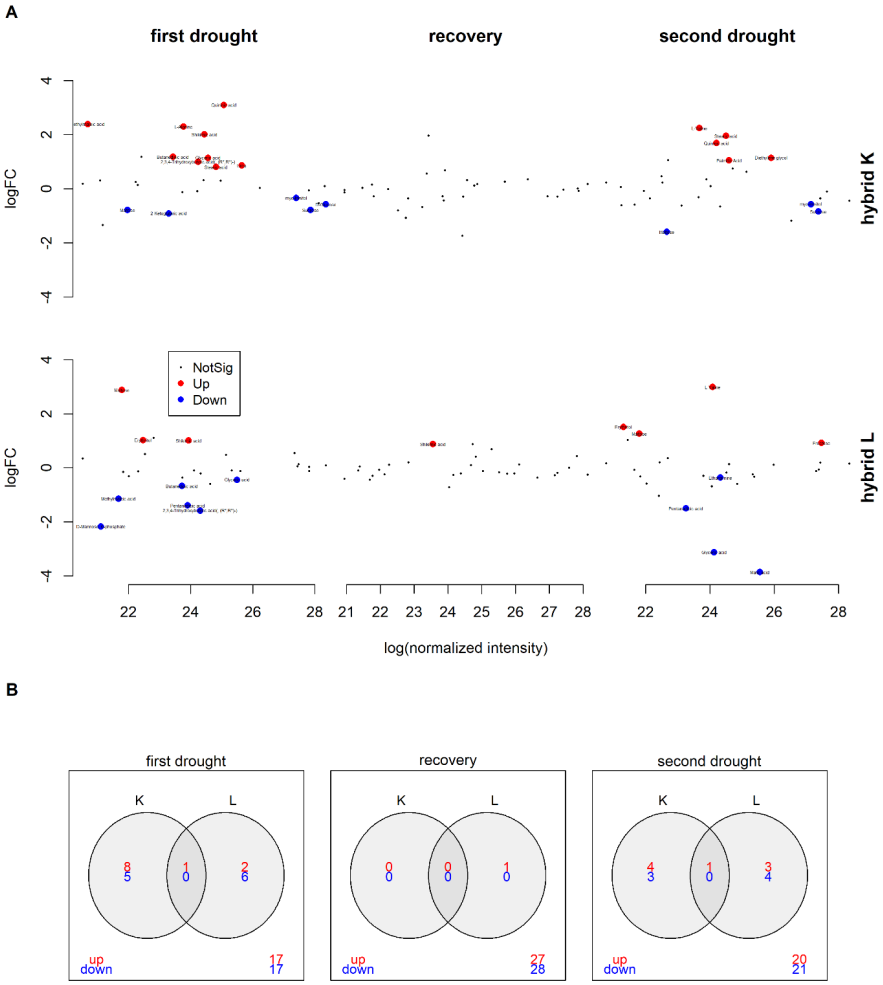

***Fig. 4**: **A,** logFC of metabolites relative to control per hybrid and time; **B,** Venn diagrams of metabolites significantly altered. Significance was determined as p.adj < 0.05.*

To investigate the metabolite reposes of repeated drought, 27 stress-responsive metabolites were measured (Fig. 4). Most significant metabolite abundance changes were detected after the first drought priming event (14 in K and 9 in L, with shikimic acid being upregulated in both hybrids). Contrasting changes occurred in butanedioic acid, 2,3,4-trihydroxybutyric acid, glyceric acid, and maltose. After recovery, all metabolite abundances returned to control values, except for shikimic acid in hybrid L. The second drought treatment caused an increase in abundance changes in both hybrids, but lower in numbers than after first drought (8 in K and 8 in L, with L-Valine being upregulated in both hybrids). Contrasting adaptations occurred in maltose. In general, significant fold changes relative to control were increased under drought but mostly returned to control values after rewatering in both hybrids (except for shikimic acid in L).

3.4 Lipid profiles and lipid remodeling

No significant differences in total lipid sets were detected after the first drought treatments in both hybrids; however, significant differences in abundance from control were detectable after the recovery from drought (Fig. 5A). The direction of fold changes to control were slightly contrasting in hybrids (0.213 up in K, -0.109 down in L). After the second drought event, a similar contrasting response trend could be observed (-0.152 down in K, 0.0845 up in L) in the repeated drought treatment, while the lipid set enrichment was not significant in any direction (up/down) of the second drought only treatment.

We then investigated patterns of lipid remodeling within lipid class sets (Fig. 5B). After the first drought, hybrid K showed downregulation of TG and PG lipids, and upregulation of LPC and sterol lipids. L showed higher PS and sterol lipids and lower TG and MGDG (and tendency of increased DGDG likely through MGDG to DGDG conversion leading to the observed increase in DGDG/MGDG ratio (Fig. 5C)). After recovery, hybrid K showed significant increases in the lipid classes of DG, LPC, CL, DGDG and MGDG and significant reduction in SQDG and sterol lipids. In contrast, L showed significant increase in PM phospholipids and reduction in DG, TG and DGDG. After the second drought in K, significant increases occurred in the lipid classes of LPE (lyso-phosphatidylerythritol), PE and PC and decreases in DG, TG, DGDG and MGDG upon the repeated drought treatment and a decrease in MGDG during the second drought only treatment. In contrast in L, only sterol was increased in the repeated drought treatment, while in the second drought only treatment, LPC and sterol increased, and MGDG decreased significantly.

The chloroplast/plasmamembrane (chloroplast/PM) logFC ratio (Fig. 5C) showed a tendency to decrease after first drought in both hybrids. After recovery, the chloroplast/PM logFC ratio was elevated in hybrid K, due to the increased synthesis of chloroplast lipids, while PM phospholipids remained at control level (Fig. 5B, K recovery). In contrast in hybrid L, the chloroplast/PM logFC ratio was further decreased after recovery, as chloroplast lipids decreased or remained at control levels, while the synthesis of phospholipids was promoted (Fig. 5B, K recovery). After second drought, the chloroplast/PM logFC ratio was decreased in both hybrids in the repeated drought treatment, but to a higher extend in K. Furthermore, the second drought only treatment displayed less change in the logFC ratio in both hybrids.

The DGDG/MGDG logFC-ratios (Fig. 5C) increased in both hybrids under first and second drought, but to a higher extent in L. After recovery, the DGDG/MGDG logFC-ratio returned to control values in tolerant hybrid L, while hybrid K produced more MGDG relative to DGDG, leading to a lower logFC ratio after recovery. After second drought, logFC ratios increased in all treatments except for repeated drought in K, indicating reduced capability to increase DGDG/MGDG ratio under repeated drought.

The PC/PE ratio remained almost constant throughout the experiment, there was a slight tendency of increased PC/PE ratio after recovery in both hybrids, and a slight reduction under drought conditions could be observed (Fig. 5C).

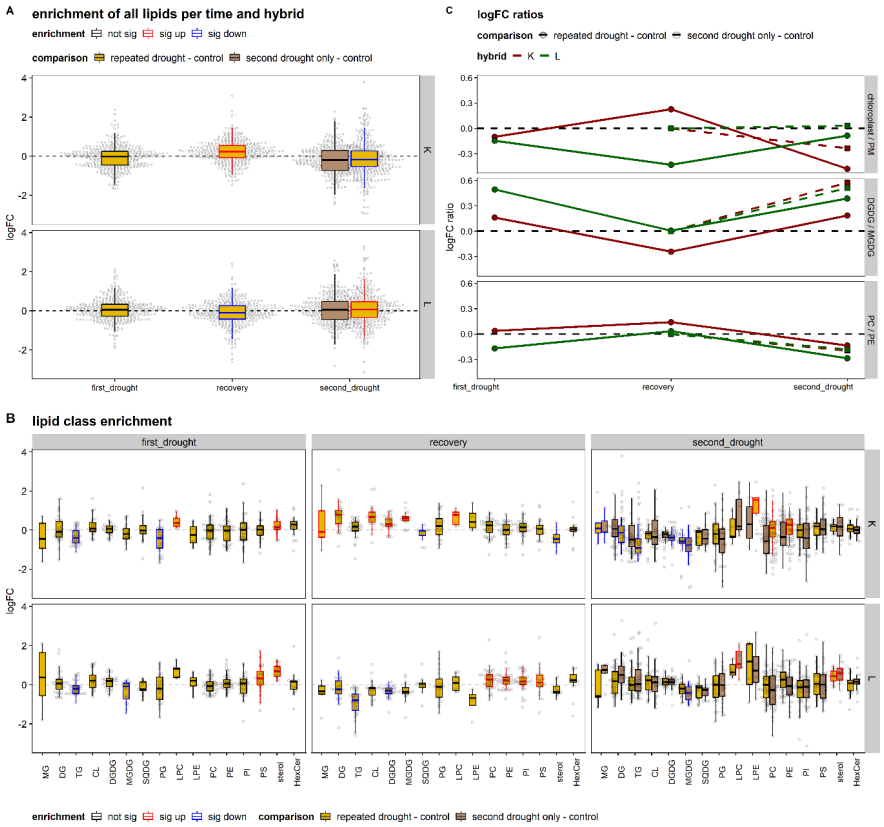

Fig. 5*: Analysis of* ***A****, lipid set enrichment of all lipids relative to control per hybrid;* ***B****, lipid set enrichment of lipid classes in the treatment groups per hybrid and experimental stage. The significance of the enrichment is indicated by the color of the boxplot (sig up/down or not sig);* ***C****, logFC ratios (i.e., logFC DGDG – logFC MGDG) in the treatment groups per hybrid and experimental stage. DGDG/MGDG logfc ratio is the ratio of the major thylakoid lipids DGDG (bilayer) to MGDG (nonbilayer). The chloroplast/PM ratio is the average logFC of chloroplast membranes (DGDG, MGDG, SQDG, PG) relative to the average logFC of plasmamembrane (PM) phospholipids (PC, PE, PI, PS). PC/PE is the ratio of the major bilayer (PC) to nonbilayer (PE) lipids in the PM.*

Next, we wanted to understand the adaption of membrane fluidity under repeated drought. We investigated the average logFC of fatty acid saturation patterns of all lipids represented as number of pi-bonds for drought treatments and the control (Fig. 6 AC) and of single lipid species (Fig. 6 BD), respectively. The levels of fatty acid saturation of all lipids at first drought were similar in both hybrids: an induction of high and low pi-bond lipids and a slight reduction of 3-8 pi-bond lipids was observed (Fig. 6A). This change was significant in the case of low pi-bonds and medium pi-bonds in L (Fig. 6A). The main difference between the hybrids became apparent during the recovery phase where hybrid K appeared to adapt by a tendency to increasing especially highly unsaturated lipids (change is not significant). On the contrary in L, medium pi-bond lipids (6-9 pi-bonds) were significantly decreased, and 1-2 pi-bonds were increased after recovery. After the second drought, both hybrids showed a decrease in 4-pi-bond lipids in the second drought only treatments (additionally in K, the 3-pi-bonds were increased). Furthermore, the 6-12 pi-bond lipids showed a tendency of increase in both hybrids after the second drought. An exception from this trend was the second drought only treatment in K, which showed no tendency of increase in the higher pi-bonds relative to control, and this could indicate a lesser potential to adjust membrane fluidity in this hybrid with previous mild drought exposure. More differences in responses became visible in the lipid class analysis (Fig. 6B): after first drought, patterns of pi-bond responses were similar in hybrids except for sterols, which displayed

higher fold change in L (Fig. 6B, first drought). After recovery, genotypic differences arose within the lipid groups of DG, TG, CL, DGDG, MGDG and LPC, which show higher logFC patterns in K (Fig. 6B, recovery). After the second drought, the patterns of the same lipid classes (DG, TG, CL, DGDG, MGDG and LPC) as well as PS and sterols showed higher logFC in L than in K (Fig. 4B, second drought). Furthermore, the second drought-only treatment in K maintained higher logFC than repeated drought in the groups of sterols, TG, DG and LPC, indicating that previous drought and recovery modulated the response to second drought in K, leading to higher decline in some lipid species, especially in TG's with 4-9 pi-bonds.

When considering lipid carbon atom index (Fig. 6 CD), the overall lipid response patterns over time resembled what was also detected for pi-bonds; a similar response after first drought in both hybrids with increasing small lipids while medium sized lipids remained around control levels, and a slight decrease in bigger lipids between 46 -70 C-atoms was observed (Fig. 6C, first drought). At recovery, hybrids diverged in responses as hybrid L showed downregulation of lipids with c-index of 46-70 while in K, c-index patterns returned to control level or were slightly upregulated in the large lipid sets > 64 C-atoms (Fig. 6C, recovery). The second drought exposure led to a decline in lipids with c-index between 46 and 74 in the repeated drought treatment in K, whereas other lipids were slightly upregulated or remain at control levels in the second drought only treatment in K and in both treatments in L (Fig. 6C, second drought).

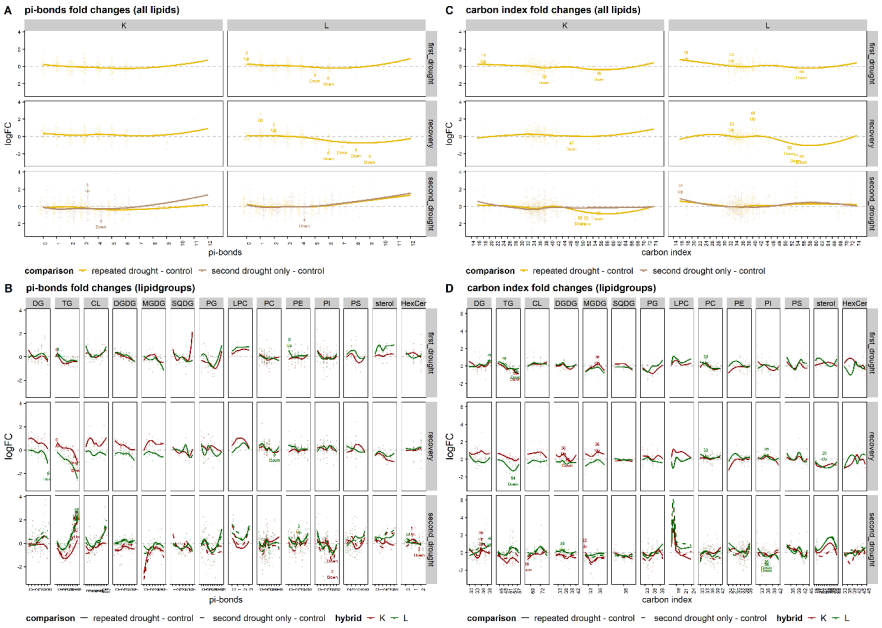

***Fig. 6**: **A, B,** Average logFC of saturation patterns of lipids represented with numbers of pi-bonds in comparison of drought treatments and the well-watered control. **A,** mean of all lipid species; **B,** single lipid species of both hybrids;**C, D,** Average logFC of lipid carbon index of the comparison of drought treatments and the control; **C,** mean of all lipid species; **D,** single lipid species of both hybrids. Lines were fitted with a loess-function. Text annotations indicate the significance of the respective set enrichment, the number refers to the lipid set which is significantly enriched (number of pi-bonds or c-index).*

4. Discussion

4.1 Maize hybrid L is more tolerant than hybrid K to repeated drought treatments

The two maize hybrids L and K display similar reductions in growth parameters, such as relative fresh weight maintenance in response to repeated drought (Fig. 2). However, differences in gas exchange, ion leakage and osmolarity were observed. Those differences suggest that hybrid L maintains a drought stress memory after

a drought stimulus, that enables it to maintain growth better when exposed to a second drought treatment. It appears that hybrid K is more sensitive to drought stress because it does not retain such a stress memory. This is in line with results obtained in a previous study investigating repeated drought in diverse maize genotypes (Kränzlein et al., 2021).

3.2 Contrasting lipid remodeling is responsible for different genotypic responses to second drought

To investigate the above-mentioned differences of both hybrids in ion leakage, we hypothesized that this discrepancy might be visible in lipid remodeling patterns, since cell membranes are early targets of oxidative stress. Comparing total lipid set enrichments there is a tendency of increased total lipid abundance under drought in the tolerant hybrid L (Fig. 3A), possibly indicating tolerance, as a decreased lipid content after drought relative to well-watered control is more common (Liu et al, 2019). In line with this tendency, sensitive hybrid K shows decreased lipid contents under severe drought (Fig. 5A). The first drought was relatively mild, such that tolerant L showed less physiological responses, while K showed higher ion leakage, osmolality and impairment of gas exchange and photosynthesis (Fig. 2). However, for the individual lipids (at p-value threshold of 0.2), hybrid L had a higher number of altered lipids than K after mild drought (24 in L vs. 3 in K, data not shown). Furthermore, unsaturation of the lipidome was increased after mild drought in both hybrids (Fig. 6A). This suggests that the lipid remodeling is elicited before cellular damage may occur (low ion leakage in L after first drought), which has been reported in some plant species (Sahsah et al., 1988) and might be an important reason for drought tolerance of L. Interestingly, the first drought event also altered the lipidome of L considerably after recovery, both in terms of singular lipid changes as well as changes in the unsaturation of the lipidome, which was reduced (Fig. 4A, recovery). This points towards a memory formation or continuous adaptation occurring in L.

Additionally, difference in lipid remodeling between hybrids is observed in the general focus on thylakoid lipids in hybrid K, while L displays a focus on phospholipids (and sterols) during the experiment, which can be observed in the chloroplast/PM logFC ratio (Fig. 5C). It seems possible that a rigidification of the PM occurs in tolerant hybrid L at the expense of photosynthetic processes, but this rigidification could be advantageous in upcoming drought. This can be observed the chloroplast lipids were less damaged relative to the PM lipids in the repeated drought treatment in L because the chloroplast/PM logFC ratio increased relative to the logFC ratio after recovery in this treatment (Fig. 5C). Conversely, the ratio of the major bilayer PM phospholipid PC and the major nonbilayer phospholipid PE remained almost constant throughout the experiment (Fig. 5C). A stable PC/PE ratio was also seen in a recent study on sorghum during salt stress (Ge et al., 2022). Furthermore, LPC and LPE are known stress signaling molecules in low concentrations but can be lipotoxic in high concentrations (Liu et a., 2019). In hybrid K, LPC and/or LPE are high in relative abundance, especially LPE under severe drought in the repeated drought treatment (Fig. 5C), which could hint for lipotoxic processes. In hybrid L, LPC is only significantly increased in the second drought only treatment, which suggests better control of lipotoxic processes in the repeated drought treatment (Fig. 5C). The adaptation strategies based on lipid remodeling are inherently different in both hybrids, showing contrasting patterns of lipid adaptation especially during the recovery phase. These differences in lipid patterns may reflect their tolerance strategies, where hybrid L achieves a more effective response towards repeated drought, while hybrid K shows a strong recovery response, which appears to be less effective towards an upcoming drought.

4.2.1 Ability to adjust DGDG/MGDG ratio and fatty acid unsaturation under drought contributes to tolerance

The DGDG/MGDG ratio is a suitable indicator for thylakoid lipid remodeling under drought and other abiotic stresses (Gigon et al., 2004; Gasulla et al., 2013). The elevated DGDG/MGDG ratio under dehydration arises mainly by a reduction in MGDG, an increase of DGDG, and conversion from MGDG to DGDG (Gigon et al., 2004; Chen et al., 2018; Du et al., 2018; Liu et al, 2019). This is thought to stabilize thylakoid bilayer structure, preventing accumulation of ROS and photodamage based on the observation, that mutants lacking MGDG conversion to DGDG and DG/TG under drought in Chlamydomonas lead to a grana hyperstacking phenotype, and since photosystem II-complexes are mostly located in the grana, while

photosystem I-complexes are mainly in the stroma, this can lead to a higher PSII/PSI ratio (Du et al., 2018). When growth is reduced by drought, the photosynthetic apparatus and photosynthetic membranes also may be reduced to prevent production of ROS by excess light harvesting activity (Du et al., 2018). It is discussed that the DGDG/MGDG ratio adjustment is an adaptation strategy rather than an indicator for stress associated damage, and the ability to increase DGDG/MGDG ratio under abiotic stress contributes to higher tolerance (Chen et al., 2018).

In this experiment, the different adjustments of the DGDG/MGDG logFC ratio between contrasting hybrids agrees with this hypothesis, as tolerant hybrid L reaches a higher ratio during both stress periods than sensitive hybrid K under repeated drought (Fig. 5B). Furthermore, the second drought only treatment in K achieved a much higher logFC ratio than repeated drought treatment, indicating that the previous drought and rewatering cycle hampered the ability of K to adjust the DGDG/MGDG ratio under drought. The reason for this could be the strong upregulation of both MGDG and DGDG after recovery (but to a higher degree MGDG, which leads to a lower ratio in K, Fig. 5B), and those lipid species are most susceptible to drought stress (Sahsah et al., 1988; Matos et al., 2002). It can be hypothesized that K has lost the ability to efficiently convert MGDG to DGDG under drought conditions, such that the excess in MGDG could not be converted, but rather was degraded through oxidative processes, similar as observed in Chlamydomonas mutants lacking MGDG conversion (Du et al., 2018). However, having high amounts of MGDG and DGDG might contribute to better growth and photosystem efficiency under well-watered conditions or at post-stress. This hypothesis is supported by the observation that assimilation rate as well as stomatal conductance in K are more sensitive under drought (Fig. 2), but recover quickly upon rewatering, leading to even higher rates of gas exchange up to 1 d after beginning of second drought. In hybrid K, the fatty acid unsaturation adaptability is higher in the second drought only treatment than in repeated drought treatment (Fig. 6A, repeated vs. second drought only). This change in fatty acid unsaturation adaptability after repeated drought vs. second drought only may indicate lipid dysregulation in K, where metabolic energy was put into over-compensation of lipids relative to control after recovery (Fig. 5AB, hybrid K recovery). However, lipid changes occurring during recovery were inefficient to deal with the subsequent drought in K better. Moreover, the fatty acid unsaturation levels were already increased before the onset of the second drought in K (Fig. 6A), suggesting the increased initial unsaturation could not be maintained under severe drought. Conversely, in hybrid L unsaturation levels were reduced after recovery but could be increased after the repeated drought (Fig. 6A, hybrid L recovery vs. second drought, repeated drought treatment, highly unsaturated lipids). A higher unsaturation of lipids is thought to increase the resistance to various abiotic stresses such as drought (Monteiro de Paula et al., 1993; Gigon et al., 2004, Zhang et al., 2005, Zheng et al., 2021) or low temperature (Vijayan, & Routaboul, 1997, Cheong et al., 2019). Therefore, it has been suggested that increased levels of unsaturation of lipids under stress is an important trait of stress tolerant plants (Ripellin et al., 1997, Gigon et al., 2004). We hypothesize that the ability to increase unsaturation under drought contributes to tolerance, and this ability was hampered in hybrid K after second drought in the repeated drought treatment.

4.2.2 Importance of the recovery state for different adaptation strategies

The recovery phase is a crucial phase where plants either keep an adapted state or reset the information obtained during drought priming (Crisp et al., 2016). The changes made during the recovery phase have a potentially high impact on the fitness of the plant towards future stress events (Hilker & Schmülling, 2019). In our experiment, significant changes with contrasting dynamics were made during the recovery period. In hybrid K, most adaptations of lipid species like MGDG, DGDG or CL occur at or after recovery and might lead to a quicker recovery of growth rates (Fig. 2, leaf elongation rate in K). However, the remodeling was ineffective to cope with upcoming drought compared with hybrid L and compared with second drought-only treatment. It is intriguing that DG is accumulated in hybrid K after recovery, but not under single drought before recovery, since DG is known to be produced during abiotic stress, and declines thereafter (Gasulla et al., 2013). One possibility is that DG serves as reservoir for energy and lipid backbone to support recovery of growth, facilitating the recovery. On the other hand, DG cannot be sufficiently produced after the second drought in the repeated drought treatment in K compared with the second drought treatment (and compared with the drought treatments in hybrid L). Additionally, as suggested above, it seems that hybrid

K could not convert the excess MGDG produced after recovery into DGDG and DG/TG under repeated drought, leading to lower DGDG/MGDG logFC ratio and therefore an impairment in adjusting chloroplast ultrastructure under drought conditions. This would cause a hyperstacking of grana which in turn might lead to production of ROS through excess light harvesting activity (Du et al., 2018), causing the inability for a directed lipid remodeling, as it occurs in hybrid L. Moreover, CL is being produced along with MGDG and DGDG during recovery in hybrid K (Fig. 5C). The drought priming could have elicited mitochondrial and chloroplast proliferation, as they synergize via a mitochondrial and chloroplast cross talk (Zottini et al., 2013), which together might lead to the strong recovery response seen in this hybrid. In general, the lipid remodeling during or after the recovery phase is crucial for explanation of the divergence of stress adaptation strategies between the hybrids. In contrast, both hybrids show a return to control conditions after recovery in terms of metabolites (Fig. 4). It can be stated that the (overshoot) lipid recovery response in hybrid K might contribute to an impairment of lipid remodeling in the upcoming drought, but this overcompensation of lipid synthesis might be beneficial for restarting growth.

4.3 Conclusion

The overall responses of two contrasting maize hybrids K and L to drought stress were analyzed with a focus on lipid remodeling. In contrast to growth, which was similarly impaired in both hybrids, ion leakage and gas exchange was mainly impaired under drought in hybrid K. We provide evidence that lipid remodeling, which is inherently different in the two hybrids, presumably plays a central role in drought adaptation. The recovery phase is the most important phase for adaptation, and changes during the recovery phase impact on fitness towards future stress. In the case of recovery-oriented hybrid K, the ability for effective lipid remodeling was reduced after recovery in the repeated drought treatment in comparison to the second drought only treatment. More specifically, the ability to increase the levels of fatty acid unsaturation and the DGDG/MGDG logFC ratio under drought are important traits, and these were hampered in the sensitive hybrid K after the first drought-rewatering cycle. On the other hand, tolerant hybrid L displayed more focus on phospholipid remodeling while efficient adjustments of DGDG/MGDG logFC ratio and unsaturation could be retained after repeated drought. Finally, hybrid L displays better control of lipotoxicity in general, but especially in the repeated drought treatment.

5. Literature

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