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Galactolipid remodeling is involved in drought-induced leaf senescence in maize

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ABSTRACT

Drought triggers leaf senescence, leading to a critical decrease in crop yield. The membrane lipid composition generally undergoes major changes during drought. However, little is known about the role of galactolipid remodeling in the drought-induced senescence response in crops. Here, a comparative study of alterations in galactolipid and fatty acid composition during drought and recovery was carried out using two maize cultivars differing in drought-induced leaf senescence. Under drought condition, the monogalactosyldiacylglycerol (MGDG) content was decreased by 18.3% and 25.9% in the cultivar with retarded leaf senescence and the senescent cultivar, respectively, while digalactosyldiacylglycerol (DGDG) was enhanced by 43.2% and 22.9%. Compared with the senescent cultivar, the DGDG content and the DGDG/MGDG ratio were 29.8% and 20.7% higher in the cultivar with retarded leaf senescence, which also maintained stable chloroplast ultrastructure under drought stress. The expressions of key galactolipid biosynthesis genes in both cultivars were up-regulated by the drought stress. In addition, the expression levels were higher in the cultivar with retarded leaf senescence than that in the senescent cultivar. These results suggests that a profound modification of galactolipid composition with high DGDG content and DGDG/MGDG ratio is associated with the alleviation of leaf senescence in response to drought. The regulation of galactolipid remodeling could be a promising strategy for alleviating the leaf senescence and improving drought adaptation in crops.

1. Introduction

Drought is the most serious environmental factor limiting the productivity of agricultural crops worldwide (Chaves et al., 2009). Predictions of future global environmental change involve increases in both severity and frequency of drought in the near future (Dai, 2013), emphasizing the urgent need to develop adaptive agricultural strategies for the changing environment. A better understanding of natural drought responses in plants would help minimize drought-related losses and ensure food production for a growing population.

Drought stress triggers various plant responses, including numerous changes in plant systems ranging from gene expression patterns to physiological metabolism and to growth and development (Fang and Xiong, 2015). One effect of drought stress is the induction of leaf senescence, which helps to reduce water loss at the whole-plant level and to remobilize nutrients from the senescing leaves to younger leaves or sink organs. Drought-induced senescence plays an important role in plant survival. However, drought-induced senescence also leads to reductions in canopy size and photosynthetic rate as well as critical decreases in crop yield after drought has been eliminated (Munne-Bosch and Alegre, 2004; Zhang and Zhou, 2013). A relationship between crop productivity and leaf senescence has been postulated for many years (Zhang and Zhou, 2013). An increasing number of reports demonstrate that delayed leaf senescence can be associated with drought tolerance and high yields under water-limiting conditions (Harris et al., 2007; Rivero et al., 2007; Degenkolbe et al., 2009; Borrell et al., 2014).

Membrane belongs to the first target of degradation during dehydration. Protection of membrane integrity is essential to maintain metabolic homeostasis (Sahsah et al., 1998). Chloroplast membranes, especially the thylakoid membranes, are believed to be highly

Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; MGD, monogalactosyldiacylglycerol synthase; DGD, digalactosyldiacylglycerol synthase

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vulnerable to drought stress associated damages (Liljenberg, 1992). Two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), are the most abundant lipids in chloroplast membrane, and constitute 70–80% of the thylakoid lipid matrix and up to 70% of the total leaf membrane lipids (Dormann and Benning, 2002; Kobayashi, 2016). During drought, changes in lipid compositions could play an important role in membrane stabilization in plants (Torres-Franklin et al., 2007). One of the widely reported drought responses is the increase in DGDG/MGDG ratio, mainly originating from the decrease in MGDG in many plant species (Stevanovic et al., 1992; Gigon et al., 2004; Torres-Franklin et al., 2007; Gasulla et al., 2013). In addition, the higher ratio of DGDG to MGDG as well as the higher galactolipid content also leads to improved salt stress tolerance in transgenic tobacco (Wang et al., 2014). Another common response to drought is the decline in the degree of fatty acid desaturation (De Paula et al., 1990; Dakhma et al., 1995). Modulated fatty acid desaturation via over expression of ω-3 desaturases in tobacco resulted in increased tolerance under both drought and salt stresses (Zhang et al., 2005). Thus, the modification of membrane lipids, especially the chloroplast lipids, could play an important role in plant stress response.

During leaf senescence, the earliest structural changes occur in the chloroplast, such as changes in the grana structure and content and formation of lipid droplet called plastoglobuli (Lim et al., 2007). Thus, chloroplasts are one of the first organelles to be targeted for breakdown (Quirino et al., 2000). The damage of chloroplast membrane is an event that occurs at an early stage during leaf senescence (Guo and Gan, 2005). The DGDG/MGDG ratio increased significantly in leaves with reference to aging and senescence (Mishra et al., 1998; Zhang et al., 2010). Recently, it has been reported that the relative contents of the MGDG decreased, while that of DGDG increased, leading to an increase in DGDG/MGDG ratio during abscisic acid- and ethylene-promoted senescence (Jia et al., 2013; Jia and Li, 2015). This increase in the DGDG/MGDG ratio may help to maintain the chloroplast membrane in the bilayer conformation which is necessary for its biological functions during leaf senescence (Jia and Li, 2015). In addition, it has been found that in the resurrection plant *C. plantagineumin*, the thylakoid membrane stays intact during desiccation, and the total lipid content remains constant while the lipid composition undergoes major changes during desiccation (Gasulla et al., 2013). These studies suggested that galactolipid modification plays an important role in leaf senescence.

Although a number of studies have been proved that both drought stress and leaf senescence can induce galactolipid changes in plant (Torres-Franklin et al., 2007; Zhang et al., 2010; Gasulla et al., 2013), little is known about the role of galactolipid remodeling in drought-induced leaf senescence, especially its role in plant resistance to drought-induced leaf senesces in major agricultural crops. Therefore, the objective of the study was to investigate the relation between the capability of galactolipid remodeling and resistance to drought-induced leaf senescence, which could contribute to a better understanding on the basic mechanisms for drought adaptation in crops. A comparative study was carried out using two maize cultivars contrasting in drought-induced leaf senescence to investigate the function of galactolipid remodeling in the drought-induced senescence response in crops. The alterations in galactolipid and fatty acid composition were studied during drought and subsequent recovery.

2. Materials and methods

2.1. Plant growth condition and drought treatment

Seeds of two maize (*Zea mays*) cultivars, cultivar LY66 with retarded drought-induced leaf senescence and senescent cultivar LY99, were sterilized with 1% sodium hypochlorite for 10 min and then washed with distilled water four times. After sterilization, six seeds were sowed per plastic pot (Diameter × Height: 300 × 290 mm) filled with 15 kg loessial soil collected from the upper 20 cm of a private cultivated field. The soil was calcic cambisols, and the soil pH value was 8.27, organic matter content was 2.4 g kg^{-1}, and total nitrogen (N), phosphorous (P) and potassium (K) content was 0.25, 0.65 and 19.2 g kg^{-1}, respectively. N, P and K (0.22, 0.15 and 0.05 g kg^{-1} dried soil) were applied as base fertilizers. The experiment was conducted in a growth chamber with a 14 h photoperiod and day/night temperatures of 28/23 °C. Photosynthetic photon flux density was 600 μmol m^{-2} s^{-1} and relative humidity was 40–50%. The plants were thinned to two per pot when the third leaves were fully expanded. When the fifth leaves were fully expanded, a natural progressive drought was imposed by withholding watering based on daily measurements of pot weight. Relative soil water content was calculated according to the weight and expressed as a percentage of maximum pot capacity (Chen et al., 2015a). In order to eliminate the effect of plant weight on soil water content, six plants of each cultivar from each treatment were measured for their individual shoot and root weight every three days, after removing the root from soil. For the control of well-watered, relative soil water content was maintained between 70% and 90% throughout the experiment; for the drought treatment, the relative soil water content was allowed to fall progressively within the first 6 days, then maintained at around 20% for 4 days by daily water supplement, and was then restored to the control level and maintained for another 4 days, as shown in Fig. 1. There were 60 pots per treatment for each cultivar, and all pots were distributed in a completely randomized design. In our preliminary experiment, the leaf senescence-related parameters and lipid contents of well-watered plants remained stable in two maize cultivars throughout the treatment period. Therefore, the photosynthetic rate, chlorophyll fluorescence, chlorophyll concentration, leaf relative water content and lipids in well-watered plants were not measured in this study. The fifth leaf of plants in drought treatment was sampled between 9:00 a.m. and 11:00 a.m. at the beginning of drought treatment (day 0, Pre-drought), the end of drought stress (day 10, Drought) and the end of recovery (day 14, Recovery). The fifth leaf tissues from three plants were pooled together for a single replicate and stored at −80 °C for simultaneous analysis of chlorophyll concentration, lipids contents and gene expression.

2.2. Growth analysis

At each time point, shoots of ten plants per treatment for each cultivar were harvested and dried in an oven at 70 °C for 72 h to determine the shoot dry weight. The relative growth rates were calculated as the percentage of increased shoot dry weight in well-watered treatment taken by that in drought treatment during drought stress and recovery, respectively (Chen et al., 2015b).

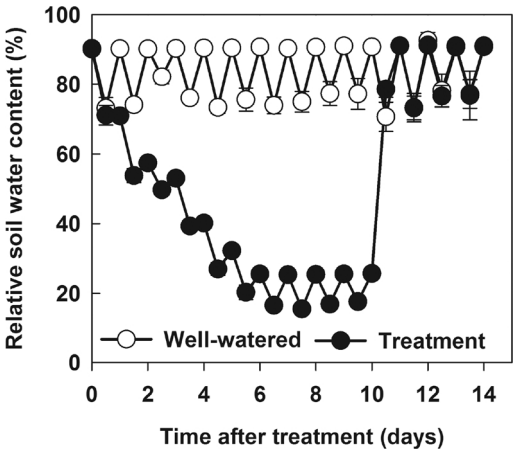

Fig. 1. Soil water content during drought stress and recovery. Data are means ± SD (n = 30).

2.3. Photosynthetic rate analysis

The fifth leaves were selected to measure the photosynthetic rate using a portable photosynthesis system (Li-6400; LI-COR Inc., Lincoln, NE, USA) between 9:00 a.m. and 11:00 a.m. The leaf was placed in a 6 cm^2 chamber at a photo flux density of 1000 μmol $m^{-2}s^{-1}$. Six plants were analyzed per treatment.

2.4. Chlorophyll fluorescence analysis

Photosystem II photochemistry efficiency (Fv/Fm) in individual leaves was analyzed using a pulse amplitude modulated chlorophyll fluorescence system (Imaging PAM, Walz, Effeltrich, Germany) according to Chen et al. (2015a). The fifth leaves were dark-adapted for 30 min before measurement. Six plants were analyzed per treatment.

2.5. Chlorophyll concentration analysis

Total chlorophyll was extracted from frozen fifth leaf samples (~0.2 g) with 80% (v/v) acetone. After centrifugation, the extract was subjected to spectrophotometric measurements (UV-2550, Shimadzu, Japan) at 645, 663, and 470 nm. The chlorophyll *a* and chlorophyll *b* concentration were calculated using the Lichtenthaler (1987) formula. Total chlorophyll concentration (mg g^{-1} DW) and chlorophyll a/b ratio were calculated. Six replicates were analyzed per treatment.

2.6. Leaf relative water content analysis

The fifth leaf of six plants per treatment were removed and weighed immediately for measurement of fresh weight (FW). Turgid weight (TW) was determined after leaf segments were immersed in distilled water for 6 h, and dry weight (DW) was measured after leaf segments were dried at 70 °C in an oven for 24 h. Relative water content (RWC) was calculated as: $RCW = \frac{FW - DW}{TW - DW} \times 100\%$.

2.7. Transmission electron microscopy for chloroplast ultrastructure

The fifth leaf tissues from four plants of each treatment were trimmed with a razor blade, fixed in 2% (v/v) glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.0) by infiltration under vacuum, and treated for 2 h. The fixed samples were washed in phosphate-buffered saline and post-fixed in 2% (w/v) osmium tetroxide in the same buffer for 2 h. They were then dehydrated in an ethanol series, transferred to propylene oxide, and embedded in Quetol 812 resin. Thin sections (70–100 nm) were cut with a diamond knife on an ultramicrotome (MT-7000; RMC), and the sections were stained with 2% (w/v) uranyl acetate for 40 min and then with 3% (w/v) lead citrate for 2–3 min. The samples were observed using a transmission electron microscope (JEM-100CX II; JEOL) at 80 kV.

2.8. Lipid analysis

Lipids were extracted according to the method of Wewer et al. (2013). Leaf sample (~0.2 g) was homogenized in liquid nitrogen with 5 mL of chloroform/methanol/formic acid (1:1:0.1, v/v/v); the homogenate was collected and shook vigorously. Subsequently, 2.5 mL of 1 M KCl in 0.2 M H_3PO_4 was added and the mixture was vortexed briefly. The homogenized samples were centrifuged at 4000*g* for 3 min, and the lower chloroform layer was transferred to a new vial. Extraction was repeated by adding 5 mL of chloroform/methanol (2:1, v/v) to the residue, shaking and centrifuging the mixture, and gathering the chloroform phase. The combined chloroform phases were evaporated with a stream of nitrogen, and 500 μL of chloroform were added. Lipids were separated by Thin Layer Chromatography (TLC) on silica gel plates (G60; Merck, Germany) according to Wang and Benning (2011). After stained with iodine, the identified MGDG, DGDG and all the remaining lipid bands (referred as other lipids, which includes phospholipids and sulfoquinovosyldiacylglycerol) were scraped off with a razor blade and placed into tubes, respectively. Then, the lipid was methylated with 1 M HCl in methanol and converted into fatty acyl methylester (FAME), and the resulting FAMEs were quantified by gas chromatography (GC-2010; Shimadzu, Japan) with flame ionization detector (FID) according to Zhang et al. (2016). Pentadecanoic acid (15:0) was used as an internal standard. The double bond index (DBI) was calculated as: DBI = [(16:1 mol% × 1) + (16:2 mol % × 2) + (16:3 mol% × 3) + (18:1 mol% × 1) + (18:2 mol % × 2) + (18:3 mol% × 3)] × 100% (Wang et al., 2010). Four replicates were analyzed per treatment.

2.9. Gene expression analysis

The fifth leaf samples were collected and frozen in liquid nitrogen to measure the transcription levels of galactolipid biosynthesis genes. The genes of galactolipid biosynthesis in maize were identified based on data from the NCBI. RNA was isolated and gene expression was analyzed using quantitative RT-PCR, as described by Liu et al. (2014), using *Actin1* as control. There were three replicates in each treatment. The genes and the sequences of their specific primers are listed in Table 1.

2.10. Statistical analysis

Data were analyzed by SPSS statistics software (Version 19.0 for Windows, SPSS, Chicago, USA). Three-way ANOVA was used to assess the main effects and interactions of cultivars, water treatments and treatment status (pre-drought, drought and recovery) for plant growth. Two-way ANOVA was used to assess the main effects and interactions of cultivars and treatment status for the rest of parameters. Differences between the mean values were compared using the least significant differences (LSD) post hoc test, and a P value < 0.05 was considered significant.

3. Results

3.1. Growth response to drought and recovery in two maize cultivars

Drought stress had a significant negative effect on biomass accumulation for both cultivars (Fig. 2A, Table S1). After recovery, the aboveground biomass was 66% in LY66 and 60% in LY99 of the well-watered plants, respectively (Fig. 2A). As shown in Fig. 2B, during 10 days of drought stress, the relative growth rate was 48% in LY66 but only 30% in LY99. And during the 4 days of recovery, the relative growth rate was 80% in LY66 but only 53% in LY99. These results showed that the cultivar LY66 had both higher drought tolerance during drought stress and greater recovery after re-watering than the cultivar LY99.

Table 1
Genes and oligonucleotides used in the real-time quantitative PCR experiment.

Gene	Accession	Primer
ZmActin1	J01238.1	F:5′-GTATGTTGCTATCGAGGCTGTTC-3′ R:5′-TCATTAGGTGGTCGGTGAGGTC-3′
ZmMGD1	NM_001148646	F 5′-CGGATGTGGGAAGTTCTCGA-3′ R 5′-GAATTCGTCTGACCTTGGGC-3′
ZmMGD2	NM_001154306	F 5′-CAGACCTCAACACATGCCAC-3′ R 5′-CGAACACGCGGATTTGAGAT-3
ZmMGD3	NM_001176586	F 5′-GGGACGAGCTCAGGAGATAC-3′ R 5′-GTCCCTGACGATGTGGAACA-3′
ZmDGD1	NM_001159060	F 5′-GACTGATGAGCACCCAAAGC-3′ R 5′-AACAATGGGCAAAGGTCGTC-3′

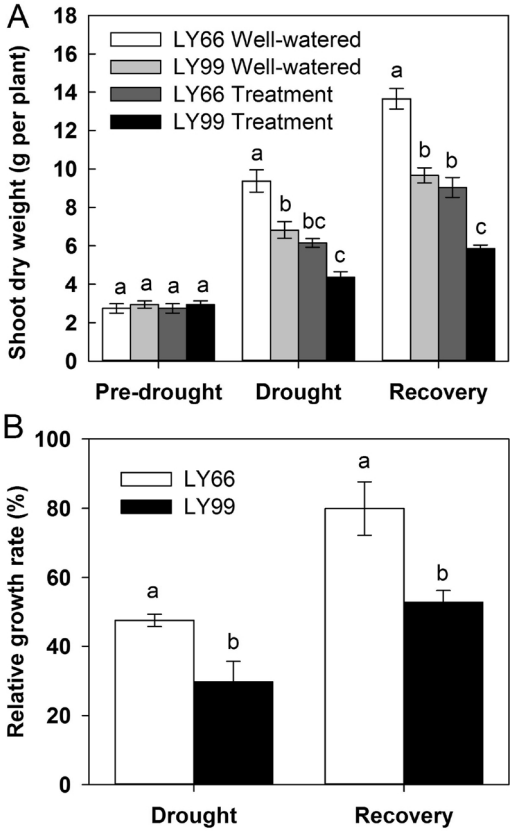

Fig. 2. Shoot dry weight (A) and relative growth rate (B) of two maize cultivars during drought and recovery. Plants were imposed to a natural progressive drought for 10 days (drought) and then re-watered for 4 days (recovery). Values are means ± SE (n = 10). Different letters indicate statistically significant differences at $P < 0.05$.

3.2. Leaf senescence response in two maize cultivars

Drought stress resulted in leaf senescence in cultivar LY99 accompanied by yellowish and withered tip leaves, but this was not the case in LY66 (Fig. 3A). The photosynthetic rate and Fv/Fm decreased significantly under drought in both cultivars, but they were higher in LY66 than in LY99 (Fig. 3B, C). After recovery, the photosynthetic rate and Fv/Fm in LY66 returned to pre-drought level, but this was not happened in LY99 (Fig. 3B, C). The chlorophyll concentration showed a substantial decrease in LY99, but no change was found in LY66 after recovery (Fig. 3D). In addition, the chlorophyll a/b ratio decreased significantly under drought in both cultivars, but LY66 still maintained higher chlorophyll a/b ratio than LY99 (Fig. 3E).

Leaf RWC was consistently and significantly reduced by drought stress and returned to pre-drought level after recovery in both cultivars (Fig. 3F, Table S1). However, no significant difference of RWC was found between two cultivars.

3.3. Effects of drought stress on chloroplast ultrastructure in two maize cultivars

As shown in Fig. 4, chloroplasts from both cultivars growing under pre-drought condition have well-developed thylakoid membrane systems composed by well-organized granal stacks and connected by stroma lamellae (Fig. 4A–D). The distortions of plastids occurred under drought stress in both cultivars. After drought treatment, the shape of the chloroplasts was approximate an ellipsoid in both cultivars (Fig. 4E, G). However, the swollen of chloroplasts and the loosen of granal stacks were more severe in LY99 than in LY66 (Fig. 4E–H). The stress-induced damage to chloroplasts and thylakoid membranes was more severe in LY99 than in LY66.

3.4. Lipid and fatty acid composition changes in two maize cultivars

The results presented in Fig. 5 showed the amounts of MGDG, DGDG, other lipids and total lipids in two cultivars. The MGDG content decreased under drought stress in both cultivars and decreased further in LY99 after recovery (Fig. 5A). The DGDG content increased substantially in LY66 but not in LY99 under drought stress and dropped sharply after recovery in both cultivars (Fig. 5B). Regarding the amount of total other lipids, it decreased under drought stress and increased back to pre-drought level after recovery in LY66, while in LY99, it decreased under both drought stress and after recovery (Fig. 5C). Moreover, all three lipids contents were higher in LY66 than in LY99 under both drought and re-watering conditions. As a result, the total lipids content was also maintained higher in LY66 than in LY99 under both drought and re-watering conditions (Fig. 5D).

In addition, the proportion (presented as moles percent of total lipids) of MGDG decreased, while the proportion of DGDG increased in both cultivars under drought stress (Fig. 6). Moreover, the DGDG/MGDG ratio enhanced greatly under drought stress and decreased after recovery in both cultivars. However, the ratio was higher in LY66 under pre-drought and drought conditions, but was lower after recovery, as compared with that in LY99 (Fig. 6D).

As shown in Fig. 7, in both maize cultivars, the main fatty acids in the MGDG and DGDG fractions were 18:2 and 18:3, which composed of 80%-90% of the total MGDG or DGDG. And the main fatty acids in the other lipids fraction were 16:0, 18:0, 18:1, 18:2 and 18:3, which composed of more than 90% of the total other lipids. Under pre-drought condition, compared with LY99, the contents of 18:3 fatty acid in MGDG and other lipids were significantly ($p < 0.05$) higher in LY66, while it was lower in DGDG, and there was no difference in total lipids between two cultivars. Under drought stress, the level of 18:3 fatty acid decreased significantly in MGDG in both cultivars, and it showed further decrease after recovery, but LY66 still maintained higher level than LY99 either under drought or after recovery. The 18:3 contents in DGDG and other lipids were not significantly affected by drought stress, but it decreased after recovery in both cultivars, and LY66 exhibited less decrease than LY99. In contrast, the level of 18:2 fatty acid increased by drought stress and showed further increase after recovery in both MGDG and DGDG fractions in two cultivars.

The DBI of MGDG and other lipids in both cultivars decreased under drought stress and decreased further after recovery (Fig. 8A, C). While in DGDG, the DBI was not affected under drought stress but dropped sharply after recovery in both cultivars. Regarding the total lipids, the DBI in LY66 was maintained constant under drought stress and decreased after recovery, as compared with pre-drought treatment; while in LY99, it decreased under drought stress and decreased further after recovery. In addition, the DBI of MGDG, other lipids as well as total lipids were higher in LY66 than in LY99 under drought treatment conditions.

The ratios of all three lipids and total lipids to chlorophyll were higher in LY66 than in LY99 under pre-drought condition (Fig. 9). Drought stress caused a substantial increase in the lipid-to-chlorophyll ratio, especially in the LY99. Thus, the ratio of all lipids-to-chlorophyll was higher in LY99 than in LY66 under drought stress. After recovery, the ratios of MGDG, DGDG and total lipids to chlorophyll dropped almost to pre-drought level, but the ratios of lipid-to-chlorophyll were still low in LY66, as compared with LY99.

3.5. Expression patterns of galactolipid biosynthesis genes

The expression patterns and the transcript levels for MGDG synthase (MGD) and DGDG synthase (DGD), two key enzymes involved in the biosynthesis of galactoglycerolipids, were analyzed. The qRT-PCR analysis showed that the expressions of all the *ZmMGD1*, *ZmMGD2*,

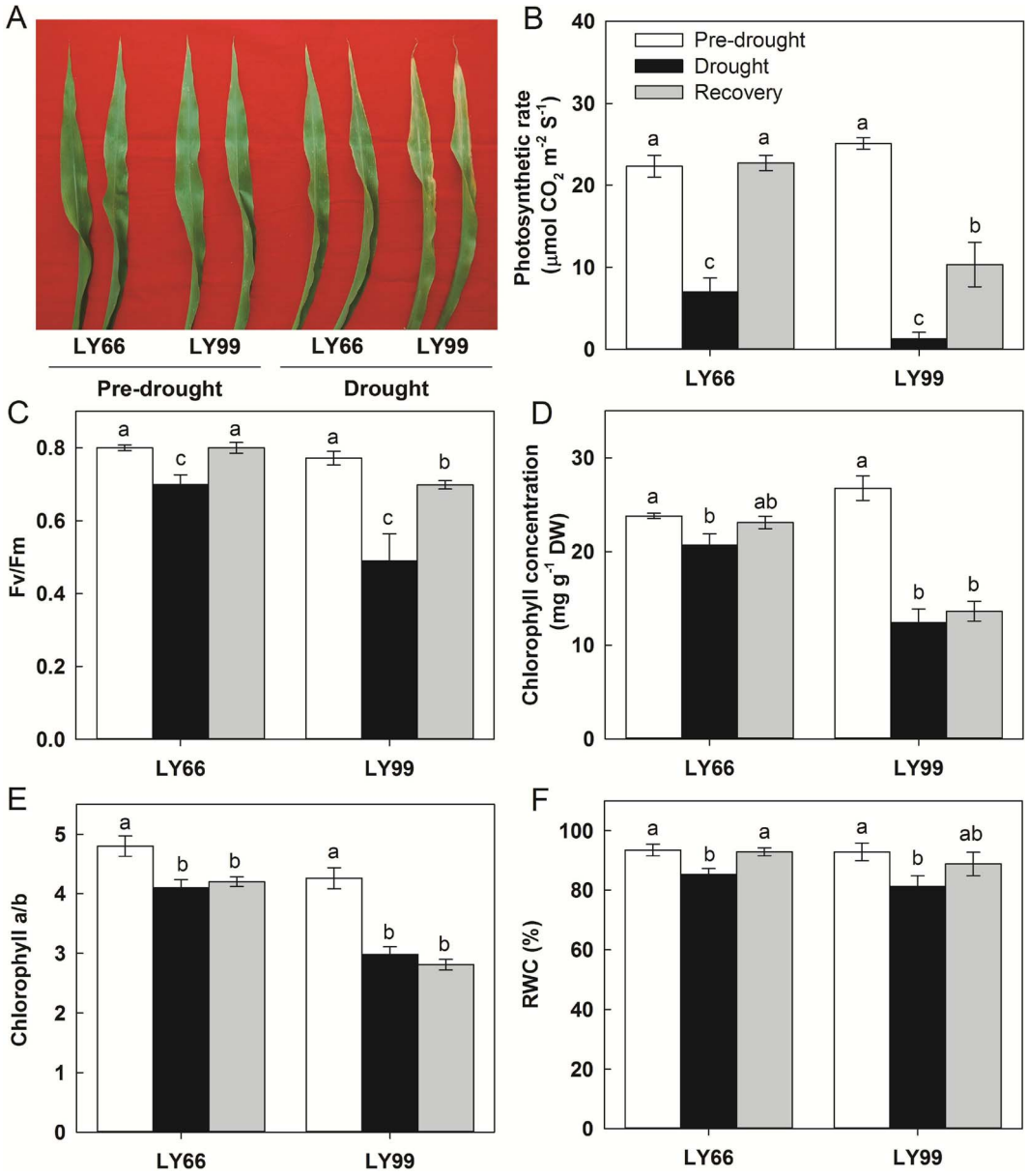

Fig. 3. Leaf senescence response and water status in the leaves of maize cultivars during drought and recovery. (A) Phenotype of leaf senescence, (B) photosynthetic rate, (C) maximum efficiency of PSII photochemistry (Fv/Fm), (D) total chlorophyll concentration, (E) chlorophyll a/b ratio and (F) leaf relative water content (RWC). Plants were imposed to a natural progressive drought for 10 days (drought) and then re-watered for 4 days (recovery). Values are means ± SE (n = 6). Different letters indicate statistically significant differences at $P < 0.05$.

ZmMGD3 and *ZmDGD1* genes were up-regulated by drought stress and recovered to the basal level after recovery in both cultivars (Fig. 10). Interestingly, the expression levels of these genes were higher in LY66 than in LY99 under both pre-drought and drought stress conditions.

4. Discussion

In the present study, the higher aboveground biomass accumulation and relative higher growth rate in LY66 than in LY99 under drought/recovery conditions indicated that the cultivar LY66 had both higher drought tolerance and drought recovery abilities (Fig. 2). Meanwhile, the LY66 showed high photosynthetic rate, Fv/Fm, chlorophyll concentration and chlorophyll a/b ratio, indicating that the cultivar LY66 displayed retarded leaf senescence induced by drought (Fig. 3). Since retarded leaf senescence generally confers high drought tolerance (Rivero et al., 2007) and the cultivar LY66 showed higher drought tolerance and recovery associated with retarded leaf senescence induced by drought. Thus, the alleviated drought-induced leaf senescence could be partly responsible for the high drought tolerance and recovery in LY66.

Drought generally modifies membrane lipid composition (Liljenberg, 1992; Gasulla et al., 2013), and the galactolipid modification plays an important role in both the adaptation to drought stress and leaf senescence response (Fourrier et al., 2008; Jia and Li, 2015). However, the relationship between the capability of galactolipid modification and the resistance to drought-induced leaf senescence is still not clear. In the present study, the DGDG, other lipids and the total lipids contents were significantly higher in LY66, in which the chloroplast ultrastructure was less destroyed, and the leaf displayed alleviated drought-induced leaf senescence, as compared with LY99 under drought stress and after recovery (Figs. 3, 4 and 5). Moreover, further comparison revealed that the profoundly modified galactolipid composition with higher DGDG content and DGDG/MGDG ratio represented the major genotypic difference (Fig. 6). These results

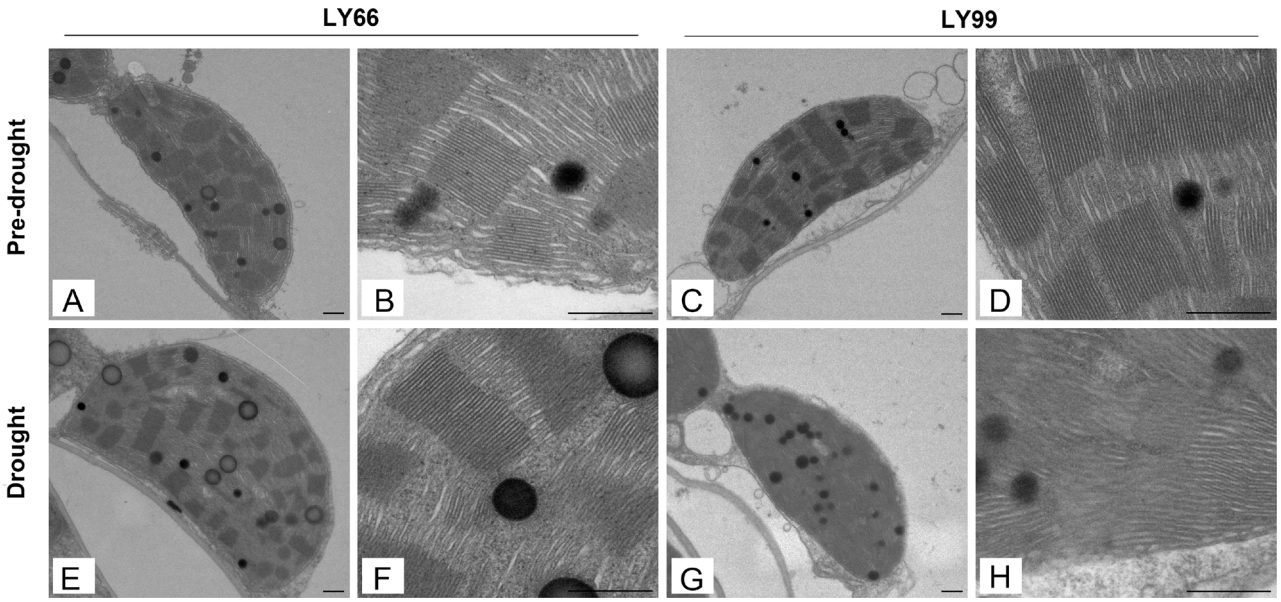

Fig. 4. Ultrastructure of chloroplasts of two maize cultivars under pre-drought and drought conditions. Electron micrographs of representative chloroplasts from LY66 (A, B) and LY99 (C, D) growing under pre-drought condition and LY66 (E, F) and LY99 (G, H) growing under drought condition are shown. In each pair of photographs (e.g. A and B, C and D, etc.), the right image represents the same chloroplast as showed on the left, but at a higher magnification. Bars = 0.5 μm.

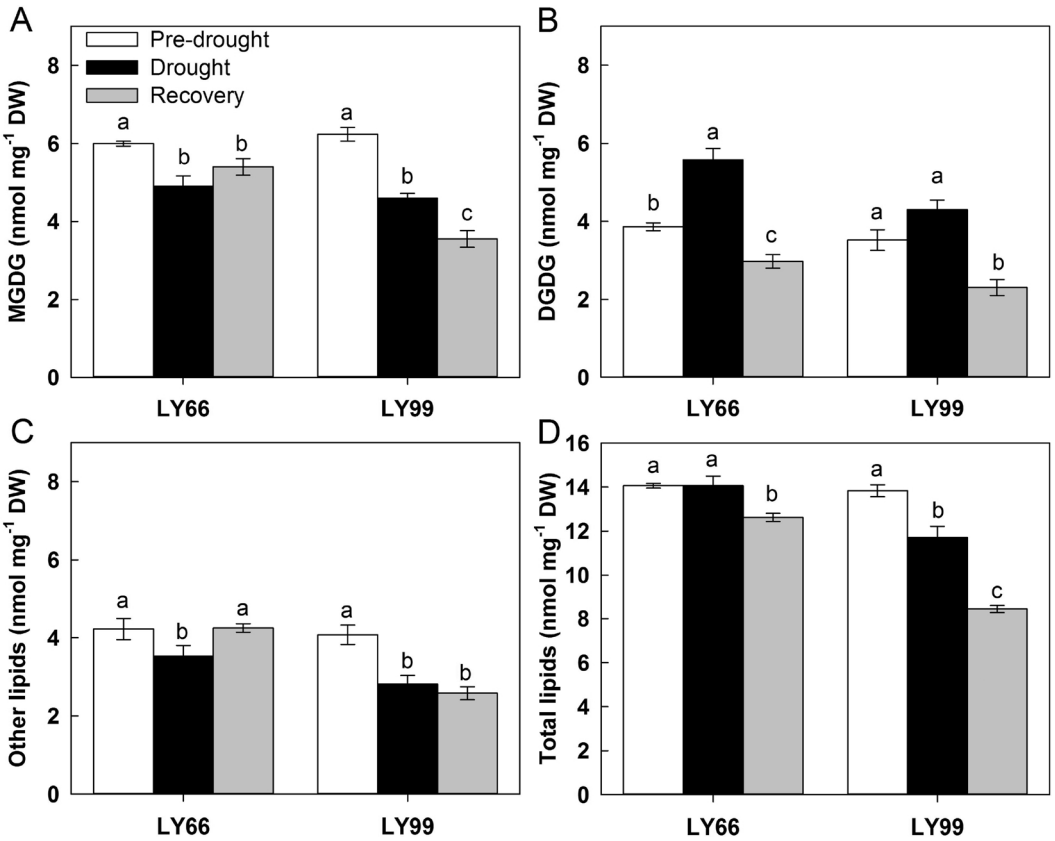

Fig. 5. Amounts of MGDG (A), DGDG (B), other lipids (C) and the total lipids (D) in the leaves of two maize cultivars during drought and recovery. Plants were imposed to a natural progressive drought for 10 days (drought) and then re-watered for 4 days (recovery). Values are means ± SE (n = 4). Different letters indicate statistically significant differences at $P < 0.05$.

suggested that modified lipid composition with higher DGDG content and DGDG/MGDG ratio was associated with the retarded leaf senescence in response to drought. Our study provided insight into the potential role of galactolipid modification in regulation of drought-induced senescence in crops.

4.1. Accumulation of DGDG contributes to the alleviation of drought-induced leaf senescence

The MGDG and DGDG comprise 70–80% of the thylakoid lipid matrix associated with photosynthetic membranes and must be regulated to maintain well organized, especially under stress conditions

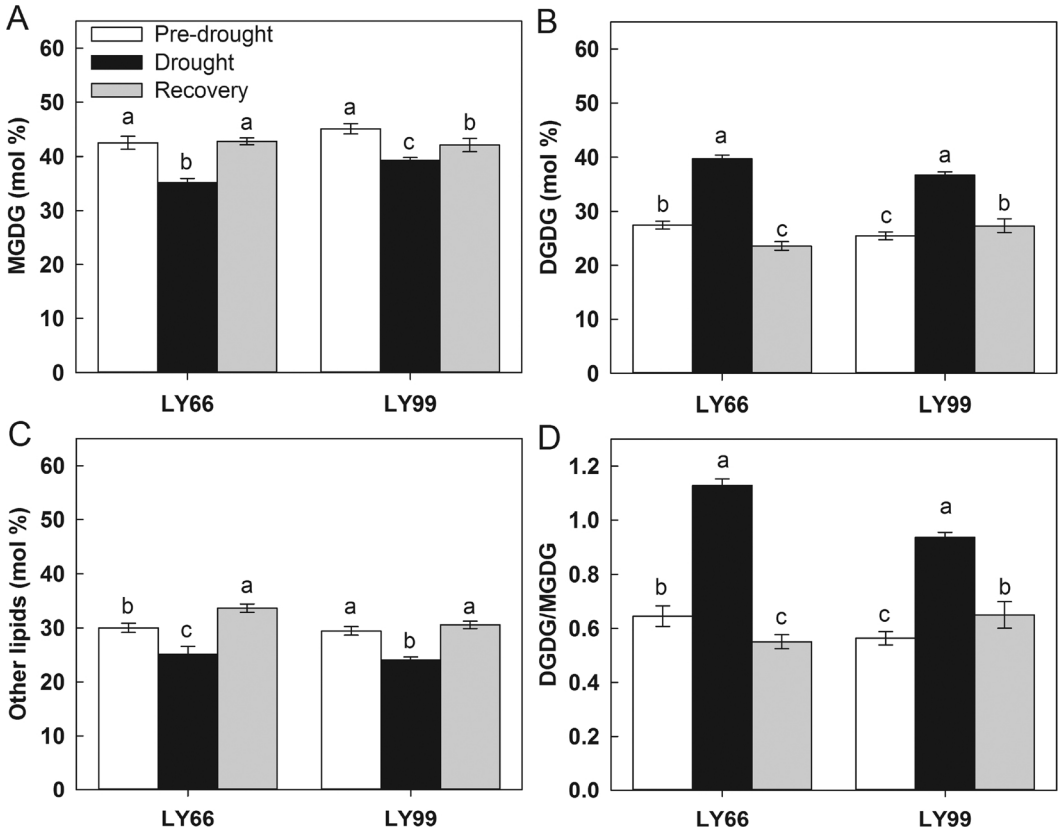

Fig. 6. Changes in proportions of MGDG (A), DGDG (B), other lipids (C) and the DGDG to MGDG ratio (D) in the leaves of two maize cultivars during drought and recovery. Plants were imposed to a natural progressive drought for 10 days (drought) and then re-watered for 4 days (recovery). Values are means ± SE (n = 4). Different letters indicate statistically significant differences at $P < 0.05$.

(Dormann and Benning, 2002; Kobayashi, 2016). The decrease in MGDG is a common adaptation to drought, osmotic stress and freezing in many plants, including cowpea, Arabidopsis and *C. plantagineum* et al. (De Paula et al., 1990; Gigon et al., 2004; Moellering and Benning, 2011; Gasulla et al., 2013). The decrease in MGDG content was also found in maize in this study. In contrast, the DGDG content increased markedly under drought stress (Fig. 6). Due to the small size of the head group, MGDG molecules are cone-shaped and non-bilayer forming, and therefore form inverted hexagonal II (H_{II}) structures, whereas DGDG are cylindrical shaped and form lamellar bilayers (Sprague, 1987; Webb and Green, 1991). Thus, the conversion of cone-shaped MGDG into cylindrical DGDG could stabilize the bilayer membrane structure during dehydration. The accumulation of DGDG also lead to an increased thickness of the head groups and an increase in the concentration of hydroxyl groups, thus enhancing the repulsive hydration forces between adjacent membranes to avoid bilayer fusion (Moellering and Benning, 2011). In the present study, the cultivar LY66 displayed alleviated drought-induced leaf senescence and also maintained higher content of DGDG than LY99 under drought stress and after recovery (Figs. 5 and 6). Similar results have been obtained previously that the DGDG content increased in tolerant cowpea cultivar, while dramatically decreased in susceptible cultivar during mild water stress (Torres-Franklin et al., 2007). Moreover, DGDG also accumulated in the desiccation tolerant plant *C. plantagineum*, in which the thylakoid membrane stayed intact and leaf avoided drought-induced senescence during desiccation (Gasulla et al., 2013). Therefore, these results suggested that the maintenance of high contents of DGDG could play an important role in membrane structural and functional stability in chloroplasts to avoid the drought-induced leaf senescence.

Although considerable amounts of MGDG and DGDG are thought to exist as “bulk lipids” and mainly function as structural lipids (Mizusawa and Wada, 2012), DGDG has also been recognized as important for the proper structure and function of PSII through specifically bounding and predominantly affecting the reaction properties of the PSII donor side (Steffen et al., 2005; Sakurai et al., 2007; Mizusawa and Wada, 2012; Kobayashi, 2016). Moreover, loss of DGDG in Synechocystis increased their sensitivity to photoinhibition under high temperature and high light, particularly by impairing the repair cycle of PSII (Mizusawa et al., 2009). DGDG deficiency also lead to enhanced photoinhibition under high light in Arabidopsis (Hölzl et al., 2009). Therefore, DGDG is required for photoprotection in both Synechocystis and plant (Kobayashi, 2016). During photoinhibition, strong light drives the photosynthetic transport of electrons, and when the quantity of electrons generated exceeds the capacity of the Calvin cycle to absorb them, the excess electrons can reduce oxygen to produce the reactive oxygen species (ROS) (Nishiyama and Murata, 2014). It has been reported that various abiotic stresses, such as salt stress, drought, heat and cold, could depress the fixation of CO_2, leading to generation of excess ROS and inhibition of the repair of PSII (Nishiyama et al., 2006; Murata and Takahashi, 2008; Takahashi and Badger, 2011; Nishiyama and Murata, 2014). Interestingly, increase of the DGDG/MGDG ratio also occurred in response to these abiotic stresses, therefore, it is possible that the increased DGDG contents also played a role in alleviating the photoinhibition under stress conditions, in addition to the structural function. Here, the cultivar LY66, which maintained a higher DGDG content and ratio of DGDG to MGDG than the cultivar LY99 under drought condition, also displayed a higher PSII activity (Fv/Fm, Fig. 3C). Thus, it is suggested that the higher DGDG content and ratio of DGDG to MGDG might contribute to alleviating photoinhibition through reducing the ROS production and photooxidative damage, and thus improve the PSII activity under drought stress and alleviate the drought-induced leaf senescence.

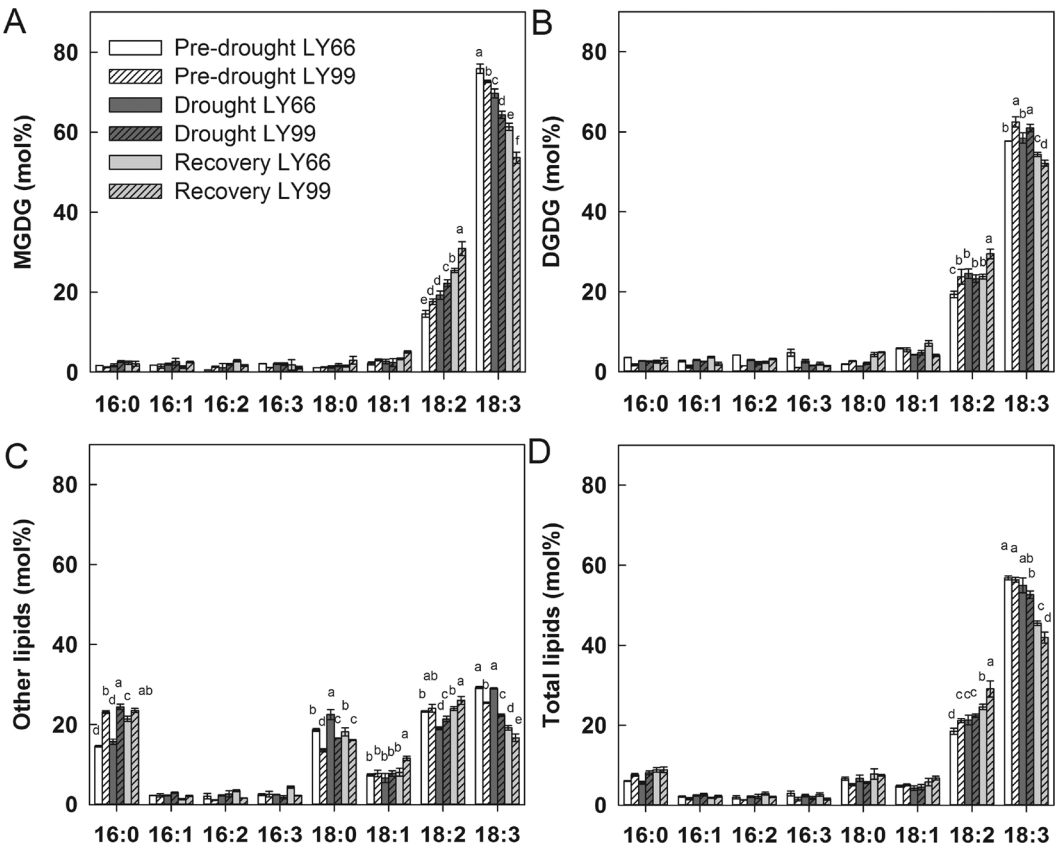

Fig. 7. Fatty acid composition of MGDG (A), DGDG (B), other lipids (C) and the total lipids (D) in the leaves of two maize cultivars during drought and recovery. Plants were imposed to a natural progressive drought for 10 days (drought) and then re-watered for 4 days (recovery). Values are means ± SE (n = 4). Different letters indicate statistically significant differences at $P < 0.05$.

4.2. Increase in DGDG to MGDG ratio is associated with the retarded drought-induced leaf senescence

In addition to DGDG content, the balance between the levels of the lamella-forming lipid DGDG and the non-lamella-forming lipid MGDG also affects the stability of thylakoid membranes (Shimojima and Ohta, 2011; Wang et al., 2014). As in all organisms, the ratio of bilayer-forming lipids to non-bilayer-forming lipids is crucial for protein folding and insertion (Gounaris and Barber, 1983; Bogdanov and Dowhan, 1999) as well as for intracellular protein trafficking (Kusters et al., 1994; Dormann and Benning, 2002). The ratio of DGDG to MGDG in chloroplasts must therefore be tightly regulated (Dormann and Benning, 2002). It is known that MGDG and DGDG are readily interconvertible (Harwood, 1998) and it is proposed that the conversion of MGDG into DGDG is a common response to dehydration (Gasulla et al., 2013). One of the well reported drought responses is the increase in DGDG/MGDG ratio (Stevanovic et al., 1992; Gigon et al., 2004; Torres-Franklin et al., 2007; Gasulla et al., 2013). Under stress conditions, the relatively high level of bilayer lipid could facilitate lamellar membrane stability. In this study, the ratio of DGDG to MGDG was increased in both cultivars under drought stress (Fig. 6 D), indicating that plants apparently intended to avoid lipid remodeling that could result in excessive membrane destabilization or in the formation of non-lamellar phases within the bilayer under drought stress. Modulation of the DGDG/MGDG ratio also occurs in response to other environmental stresses, such as phosphate starvation, heat stress and freezing. It has been demonstrated that the MGDG produced by type B *MGD* gene was thought to be used directly for subsequent DGDG synthesis in Arabidopsis under phosphate starvation (Kobayashi et al., 2009; Shimojima and Ohta, 2011). In addition, increase in DGDG/MGDG ratio is also widely believed to enhance the stability of the thylakoid membrane at elevated temperatures (Süss and Yordanov, 1986; Chen et al., 2006). In this study, the cultivar with retarded leaf senescence maintained a high ratio of DGDG/MGDG under drought condition, giving them a high capability to stabilize and maintain their membrane structures and functions, as well as maintained a high content of chlorophyll; this could explain how the cultivar with retarded drought-induced leaf senescence maintained high photosynthetic rate under drought stress, at least partially.

Under stress conditions such as nitrogen deficiency and salt stress, the lipid-to chlorophyll ratio is markedly increased, indicating that the protein-packing density in thylakoids tends to be decreased under stresses (Gaude et al., 2007; Wang et al., 2014). In this study, the damage to the chloroplast membrane and the decrease in chlorophyll concentration were less in the cultivar with retarded leaf senescence, while the protein-packing density was significantly high (lower lipid-to-chlorophyll ratio) under drought stress (Fig. 9); all those changes may be ascribed to their higher DGDG content and DGDG/MGDG ratio under drought stress (Fig. 8). The increase in DGDG/MGDG ratio could be an adaptive strategy of plants to alleviate the drought-induced leaf senescence by maintaining the membrane in a physical state compatible with normal functioning of membrane proteins.

4.3. Accumulation of DGDG is associated with MGD and DGD expression

Changes in lipid contents might be caused by alterations in the expression of genes involved in lipid biosynthesis or degradation. Two key enzymes are involved in the biosynthesis of these galactoglycerolipids: MGDG synthase, which transfers a galactosyl residue from UDP-Gal to diacylglycerol, and DGDG synthase, which catalyzes the further galactosylation of MGDG to form DGDG (Shimojima and Ohta, 2011). In this study, the transcript levels of *ZmMGD1*, *ZmMGD2*, and *ZmMGD3*

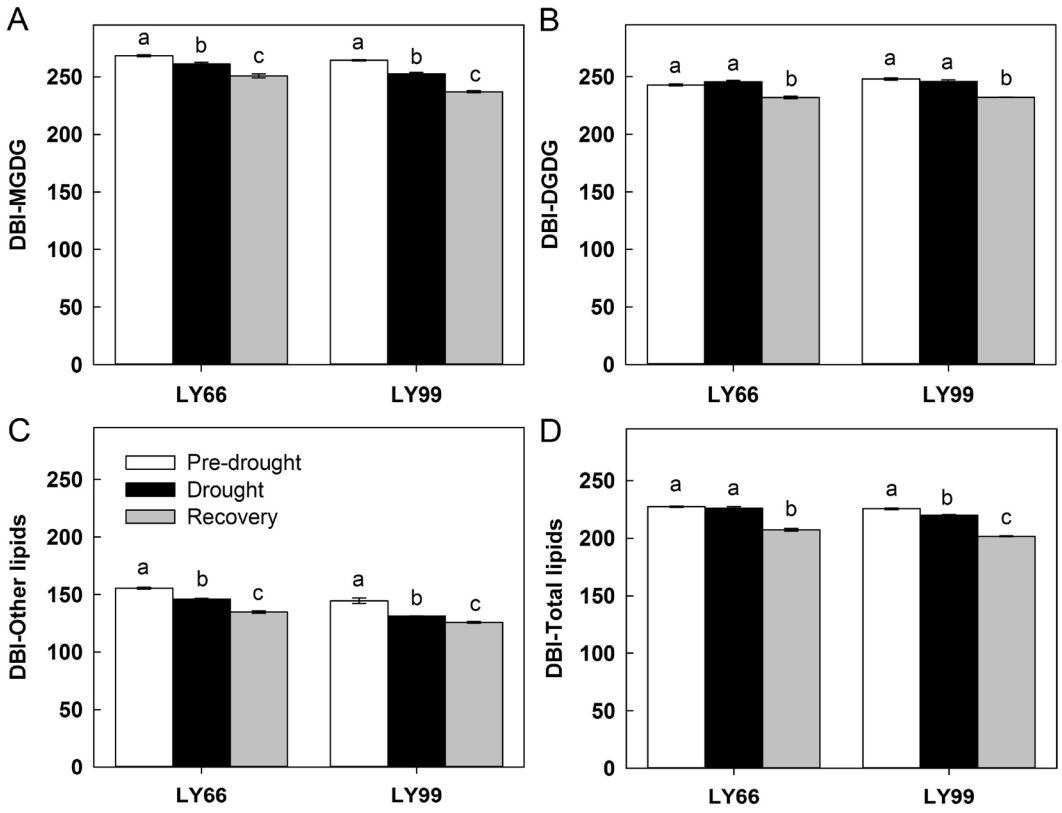

Fig. 8. The double bond index (DBI) of MGDG (A), DGDG (B), other lipids (C) and the total lipids (D) in the leaves of two maize cultivars during drought and recovery. Plants were imposed to a natural progressive drought for 10 days (drought) and then re-watered for 4 days (recovery). Values are means ± SE (n = 4). Different letters indicate statistically significant differences at $P < 0.05$.

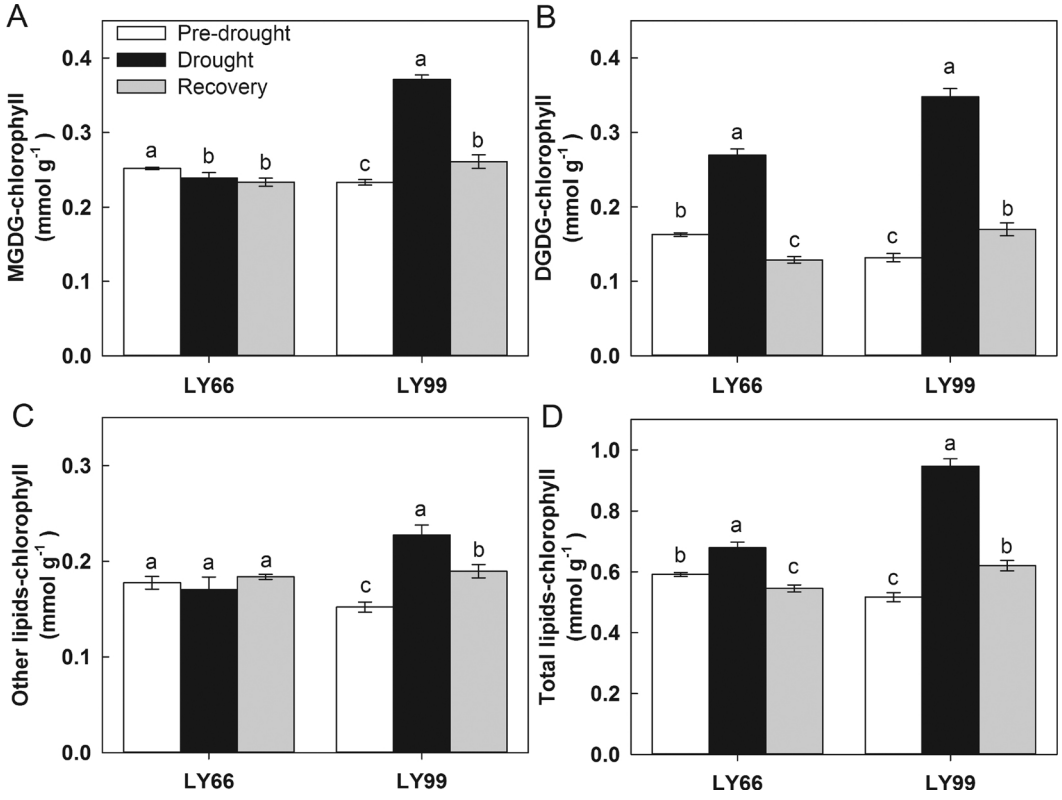

Fig. 9. Lipid-to-chlorophyll ratio of MGDG (A), DGDG (B), other lipids (C) and the total lipids (D) in the leaves of two maize cultivars during drought and recovery. Plants were imposed to a natural progressive drought for 10 days (drought) and then re-watered for 4 days (recovery). Values are means ± SE (n = 4). Different letters indicate statistically significant differences at $P < 0.05$.

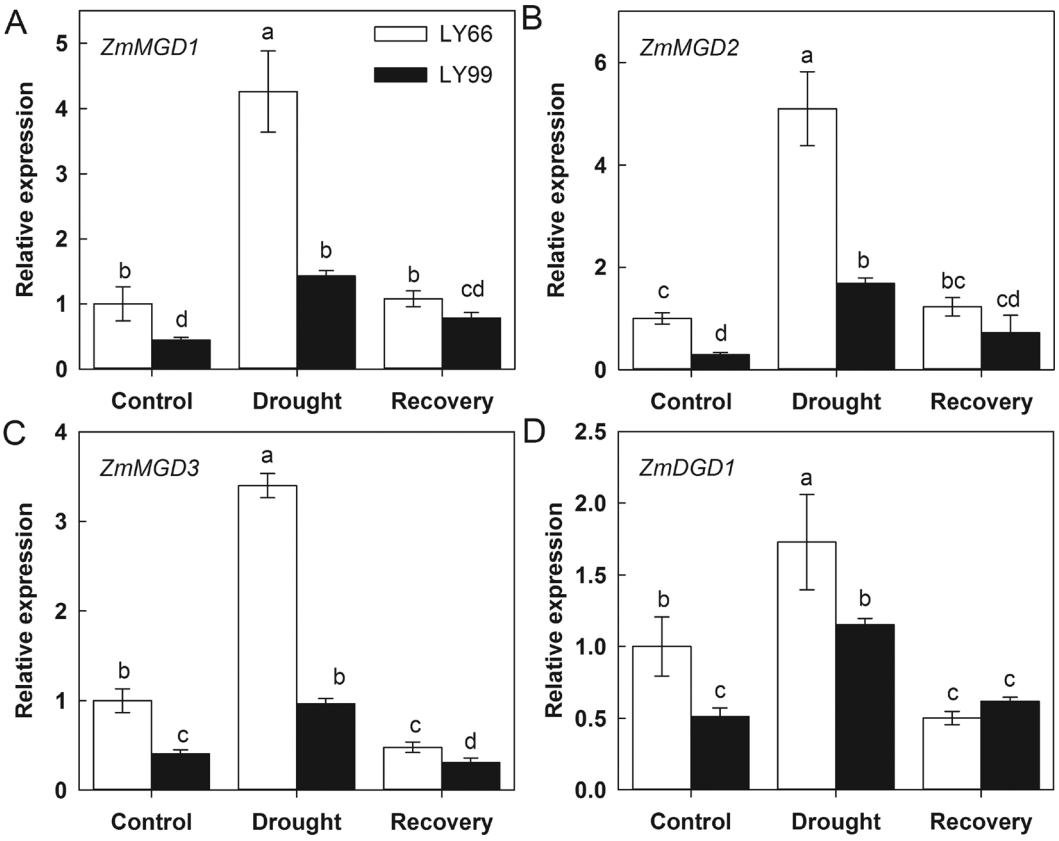

Fig. 10. Expression of galactolipids biosynthesis genes in the leaves of two maize cultivars during drought and recovery. (A) *ZmMGD1*, *monogalactosyldiacylglycerol synthase1*; (B) *ZmMGD2*; (C) *ZmMGD3*; (D) *ZmDGD1*, *digalactosyldiacylglycerolsynthase1*; Plants were imposed to a natural progressive drought for 10 days (drought) and then re-watered for 4 days (recovery). Values are means ± SE (n = 3). Different letters indicate statistically significant differences at $P < 0.05$.

and *ZmDGD1* in both cultivars were up-regulated by the drought stress and recovered to the basal expression level after recovery (Fig. 10). These results showed that the alterations in the expression of *MGD* and *DGD* genes could be responsible for the accumulation of DGDG under drought stress. The observed discrepancies between expression of *MGDs* and MGDG content could be the result of a balance between biosynthesis and degradation. Moreover, the newly synthesized MGDG by *MGD* might be catalyzed into DGDG by *DGD* under drought stress. It is known that environmental stresses such as drought stimulate the galactolipase activities and gene expression (Ferrari-Iliou et al., 1994; Matos et al., 2001; Torres-Franklin et al., 2007). In addition to the DGD pathway, the glycolipids: glycolipids galactosyl transferase SFR2 can also produce DGDG by transferring a galactose from one MGDG to another (Moellering and Benning, 2011). However, it is reported that in desiccated *C. plantagineum*, the outermost galactose (Gal_{II}) in DGDG was in the α-configuration, indicating it was synthesized by DGD pathway (Gasulla et al., 2013). Thus, the accumulation of DGDG under drought stress perhaps mainly results from the stimulated MGD and DGD activities along with the gene expression. Moreover, the expression level of *MGD* and *DGD* genes was higher in retarded leaf senescence cultivar than in senescent cultivar under drought stress, which might be responsible for the higher DGDG content in the cultivar with retarded leaf senescence under drought stress.

During severe drought stress, the decline in the membrane lipid contents has been widely reported (Liljenberg, 1992; Toumi et al., 2008). This decline mainly results from the activation of degradation progresses, such as lipid hydrolysis by hydrolytic enzymes (Sahsah et al., 1998) and peroxidation caused by ROS (Ferrari-Iliou et al., 1994). Kaoua et al. (2006) showed that the galactolipase and phospholipase activities were stimulated in stressed and senescing plants, and Ferrari-Iliou et al. (1994) also reported that the peroxidation of thylakoid lipids increased under drought condition. In the present study, the decline of galactolipid content occurred in the cultivar LY99, whereas the galactolipid level remained stable in the cultivar LY66 which showed retarded leaf senescence (Fig. 5). Similar results have been previously reported in various plants with different drought tolerance (Torres-Franklin et al., 2007; Toumi et al., 2008). Therefore, the decline of galactolipid content in cultivar LY99, in which the onset of leaf senescence was induced by drought, might be explained by an activation of lipid degradation processes.

In addition, the decline in the degree of fatty acid desaturation is another common response to dehydration (Depaula et al., 1993; Dakhma et al., 1995). The decrease in fatty acid desaturation under stress conditions is disadvantageous to the stability of the thylakoid membrane because it tends to reduce the fluidity of the thylakoid membrane (Karim et al., 1999; Zhang et al., 2005; Sui et al., 2010; Wang et al., 2010). A significant decrease in fatty acid desaturation has also been observed during leaf aging (Zhang et al., 2010). It has been reported that modulated fatty acid desaturation via over expression of ω-3 desaturases in tobacco resulted in increased tolerance to salt and drought stress (Zhang et al., 2005). In this study, the cultivar LY66 maintained a higher degree of fatty acid desaturation than the LY99 did under drought condition, giving them a high capability to stabilize the membrane fluidity (Fig. 8). This result suggests that maintaining high degree of fatty acid desaturation may also play an important role in membrane stability to alleviate the drought-induced leaf senescence. Moreover, the decline in the degree of fatty acid desaturation might be attributed to the peroxidation of unsaturated fatty acids by ROS, which increased under drought stress (Ferrari-Iliou et al., 1994; Kaoua et al., 2006) and in senescing leaves (Zhang et al., 2010). In addition, the lipophilic membrane-bound chloroplastic antioxidants, carotenoids and tocopherols, are essential for protecting chloroplast membranes from

oxidative damage (Munné-Bosch et al., 2001). Here, the cultivar LY66 could maintain a higher degree of fatty acid desaturation and also had a higher carotenoids content than LY99 (data not shown). Thus, it is possible that the cultivar LY66 had a stronger chloroplastic antioxidant defense ability, which helped it to maintain high degree of fatty acid desaturation under drought stress.

In conclusion, the different leaf senescence responses to drought in two maize cultivars could be associated with the capability of galactolipid remodeling. The increased DGDG level and DGDG/MGDG ratio through the stimulated expression of the *MGD* and *DGD* genes may play an important role in conferring alleviated drought-induced leaf senescence. This could provide a novel strategy for drought adaptation in plants and crops.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.envexpbot.2018.02.017.

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