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Changes in chloroplast ultrastructure in leaves of drought-stressed maize inbred lines

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Abstract

Chloroplasts are commonly the site of the earliest abiotic injury visible in plant ultrastructure. In this study, six inbred lines of maize (*Zea mays* L.) were used to analyze changes in the ultrastructure of chloroplasts and related physiological parameters under conditions of drought stress simulated by 20% polyethylene glycol 6000 (–0.6 MPa) for two days. Chloroplasts of three maize lines proved to be more sensitive. They showed changes in the ultrastructure in response to drought, including damage of thylakoid membranes, an increase in the number and size of plastoglobuli, swelling of thylakoid membranes both stromal and granal, disorganization of the thylakoid membrane system, an obvious increase in the intrathylakoid space, and a decrease in the length-to-width ratio and area of chloroplasts. In addition, the contents of malondialdehyde increased markedly in the sensitive lines. Contrary to the sensitive lines, stable structures and shapes of chloroplasts were observed in the drought-resistant lines; it could be considered as an advantage contributing to drought tolerance in the plants. In addition, the drought index of leaf fresh mass (LMDI) in the drought-sensitive lines was ≤ 0.5, which was also associated with a lower content of leaf chlorophyll. In contrast, drought tolerance coincided with lesser growth reduction, and higher LMDI and leaf chlorophyll content.

Additional key words: biomass; chlorophyll; maize; membrane peroxidation; water content.

Introduction

Environmental stresses including drought, temperature extremes, heavy metals or salinity can severely impair plant growth and development. Of the various abiotic stresses, drought is considered to be a major environmental constraint and it limits crop productivity (Farooq *et al.* 2008, Kallis *et al.* 2008). Maize is the most important crop in China, and the third most important worldwide. It is also considered as one of the most drought-sensitive crops: it is estimated that 15–20% of the grain yield of maize is lost each year due to drought; such losses may increase further as droughts become more frequent and severe because of climate change (FAOSTAT 2010).

The contribution of photosynthesis to crop productivity under drought stress has received much attention worldwide in recent years. Chloroplasts are the sites of photosynthesis and the first organelles of mesophyll cells to be affected by drought stress (Noodén *et al.* 2004). Any change in their ultrastructure is also associated with changes in photochemical activity (Rehem *et al.* 2011). Thus, structure of leaf chloroplasts is suggested to be an important general trait related to plant drought susceptibility (Vassileva *et al.* 2012). Under the conditions of severe drought, mesophyll cell ultrastructure is disturbed, which leads to broken membrane systems in chloroplasts, swollen grana, and degraded starch granules. Cristae of mitochondria become severely disorganized and the matrix shows a lower electron density (Xu *et al.* 2009). Gradual water stress may cause more controlled changes in the configuration of cell organelles, which contributes to their stability and higher drought tolerance. In addition, leaf cell organelles are better preserved in drought-tolerant compared to sensitive genotypes. It has also been reported

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Abbreviations: DI – drought index; DM – dry mass; FM – fresh mass; G – granal thylakoids; LFMC – leaf fresh matter of control plants; LFMD – leaf fresh matter of plants under drought stress; LMDI – drought index of leaf fresh mass; MDA – malondialdehyde; P – plastoglobuli; PEG – polyethylene glycol; ROS – reactive oxygen species; RWC – relative water content; SG – starch grain; ψ_w – water potential.

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that chlorophyll (Chl) contents might estimate the influence of environmental stress on photochemical activity and could be used as an index for evaluating plant drought tolerance (Murchie and Lawson 2013).

There is a growing demand for breeding of new crops with stable and high yields under conditions of low water availability. Phenotyping for drought tolerance is laborious and resource intensive, requiring multiple locations and years of field testing to accurately characterize the traits involved. Thus, efficient breeding for drought tolerance requires an easy and manageable screening system to improve the selection process, because field trials are time- and labor-demanding (Fulda *et al.* 2011). The use of a hydroponic approach as an alternative has been much discussed (Verslues *et al.* 2006), especially, because it achieves high reproducibility under standardized conditions that cannot be attained in field trials. According to Verslues *et al.* (2006), polyethylene glycol (PEG) is the best solute for simulating low water potentials (ψ_w) that reflect the type of stress imposed by drying soil (van der Weele *et al.* 2000, Verslues and Bray 2004); this suggests that PEG simulation might be useful in screening and selecting for drought responses of plants, prior to field trials.

The evaluation and selection of drought resistance in inbred lines is one of the biggest challenges to breeders of maize. The potential applications of morphological traits and structure of mesophyll cells in breeding for drought tolerance in important plant species (*e.g.*, *Arabidopsis* and wheat) were reviewed by Valerio *et al.* (2011) and Vassileva *et al.* (2012), yet reports on alterations of drought-resistant traits of maize inbred lines in hydroponic culture are limited. The aim of the present study of drought tolerance was to assess the response of chloroplast ultrastructure to PEG-simulated drought stress using six inbred lines in order to investigate the mechanisms behind drought tolerance in maize.

Materials and methods

Plant material and culture conditions: Hydroponic experiments were carried out in April 2013. Seeds of six inbred lines of maize were obtained from the Agronomy College of Henan Agricultural University (Zhengzhou, China) and the Agronomy College of Northwest A & F University (Shaanxi, China). Three lines (CML288, HZ4, and Y82) are the key inbred lines in China, while the others (M800, M265, and Wi6-2) are newly developed. The seeds were soaked in water containing 0.2% (w/v) thiuram and benomyl for 24 h to prevent fungal infection, then placed on moistened gauze spread across disks, and finally kept in darkness to germinate at 28°C for 4 d. Uniformly germinated seeds were planted in pots filled with clean quartz sand and cultured with the whole Hoagland's nutrient solution in a growth chamber (*LT/ACR-2002*, *Yishengtaihe Com.*, China) for 20 d under a 13-h photoperiod, 28/22°C of day/night temperature, light intensity of 600 μmol(photon) m^{-2} s^{-1}, and relative humidity of 60/75% (day/night).

PEG stress: Maize seedlings grew for 20 d, then 20% PEG (–0.6 MPa) was added to the Hoagland solutions and the seedlings were grown for next 2 d. Three replicates of each treatment were randomly arranged and repeated four times.

Growth parameters: Two days after application of PEG stress, the plant growth was characterized by measurement of height and fresh mass allocation in the leaves of ten plants. The samples were placed in an oven at 105°C for 15 min, and then dried to a constant mass at 75°C. Leaf water content was calculated using the equation: leaf relative water content (RWC, %) = (fresh mass – dry mass) × 100/(saturated mass – dry mass) (Li *et al.* 2001). Drought index (DI) of leaf growth based on fresh matter was calculated using the following relationship (Hunt and Rock 1989):

$$\text{LMDI} = \text{FMD}/\text{FMC}$$

where FMC is the average leaf fresh matter of control plants, FMD is the average leaf fresh matter under drought stress, and LMDI is the drought index of leaf fresh mass.

Lipid peroxidation: Plant material was homogenized with 3 mL of 0.5% thiobarbituric acid in 20% trichloroacetic acid (w/v). The homogenate was incubated at 100°C for 30 min and the reaction was then stopped in ice or cooled water. The samples were centrifuged at 10,000 × *g* for 10 min and absorbance was recorded at 450, 532, and 600 nm using a *UV-1800* spectrophotometer (*Shimadzu Corp*., Japan). The MDA concentration was determined by the following equation:

$$C_{MDA}\ (\mu mol\ L^{-1}) = 6.45\ (A_{532} - A_{600}) - 0.56A_{450},$$

from which the absolute concentration [μmol g^{-1}(FM)] of MDA was calculated (DeLong *et al.* 2002).

Chl pigment was extracted in 95% ethanol in darkness for 24 h. Afterwards, the sample was centrifuged for 10 min at 8,000 × *g*. The supernatant was collected and absorbance read at 665, 649, and 470 nm using a *UV-1800* spectrophotometer (*Shimadzu Corp*., Japan). The contents of Chl and carotenoids (Car) were calculated using the equations of Lichtenthaler and Buschmann (2001).

Transmission electron microscopy: To avoid structural difference in different parts of leaves, the middle portion of leaves without the midrib was used. Each portion was cut into small pieces (about 0.1–0.5 cm) that were placed in a bottle with 4% glutaraldehyde buffer solution. Air was pumped out of the bottle with a syringe in order to soak leaves fully in buffer solution according to Chen (2004), with some modifications. Leaves were fixed at 4°C for 24 h. They were then rinsed in phosphate buffer (pH 7.4) for 15 min and post-fixed in 5% OsO_4 at room temperature. The fixed samples were dehydrated in an ascending serie of ethanol dilutions (50, 60, 70, 80, and 90%; 15 min each) and in 100% ethanol (three times for 7–8 min), washed in 100% acetone for 15 min, and embedded in *Epon 812* resin. Thin sections were cut with an *LKB-V* ultramicrotome (*LKB*, Sweden) and double stained with uranium acetate–lead citrate before being examined under a transmission electron microscope (*600-A-2, Hitachi*, Japan) operating at 75 kV.

Morphometric and statistical analyses: The image analyses were performed using the software *Image J, version 1.36b* (*National Institute of Health,* USA) and *Cell B* (*Olympus,* Germany). The size of chloroplasts was measured, and the number of organelles was determined per 100 μm^2 of mesophyll cell area, and at least fifteen chloroplasts from each treatment were examined.

The results presented are the mean with standard deviations of at least three independent experiments. Data were analyzed with a multifactor analysis of variance (*ANOVA*) using the *Statgraphics Plus v.4.1* software, and statistical significance was defined as $p<0.05$ or $p<0.01$.

Results

Effect of drought stress on growth parameters of maize seedlings: The plant FM declined significantly in the lines CML288, M800, M265, Y82, Wi6-2, and HZ4, by 7.8, 24.6, 25.2, 35.5, 42.8, and 39.1%, respectively (Fig. 1*B*). CML288, M800, and M5212 showed higher drought resistance indices of leaf growth (LMDI = 0.72, 0.57, and 0.56, respectively), and no significant differences were found in plant height under drought stress compared with controls (Fig. 1*A*,*D*). In addition, the Chl contents of leaves in CML288 and M800 increased significantly by 8.8 and 29.8 %; in Wi6-2 and HZ4, they were reduced by 21.2 and 29.8%, respectively (Fig. 1*C*).

Effect of drought stress on MDA and water content of maize seedlings: After 2 d of drought stress, there were no significant differences in MDA content of CML288, M800, and M265 seedlings, but the MDA content increased significantly in Y82, Wi6-2, and HZ4 by 34.9 %, 32.4%, and 59.6%, respectively (Fig. 2*A*). RWC in CML288, M800, and M265 decreased insignificantly, but it decreased markedly in Y82, Wi6-2, and HZ4, by 17.3, 19.5, and 25.4%. These results combined with growth

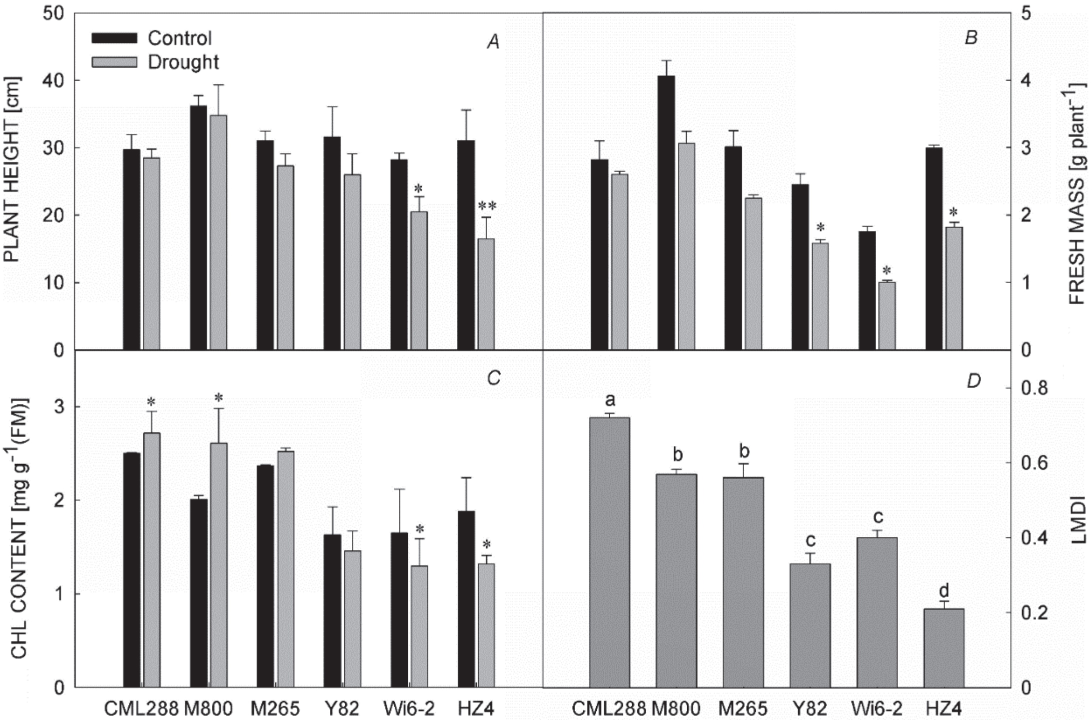

Fig. 1. Effects of drought stress on plant height (*A*), fresh mass of roots + shoots (*B*), chlorophyll (Chl) content of leaves (*C*), and drought index of leaf fresh mass (LMDI) (*D*); 20 d-old seedlings of maize inbred lines were treated with 20% PEG solution (–0.6 MPa) for 2 d. Bars represent standard errors of triplicate experiments. *Different letters* indicate significant differences at $p<0.05$; * or ** indicate significant differences between control and drought stress treatments at $p<0.05$ and $p<0.01$, respectively (one-way *ANOVA*).

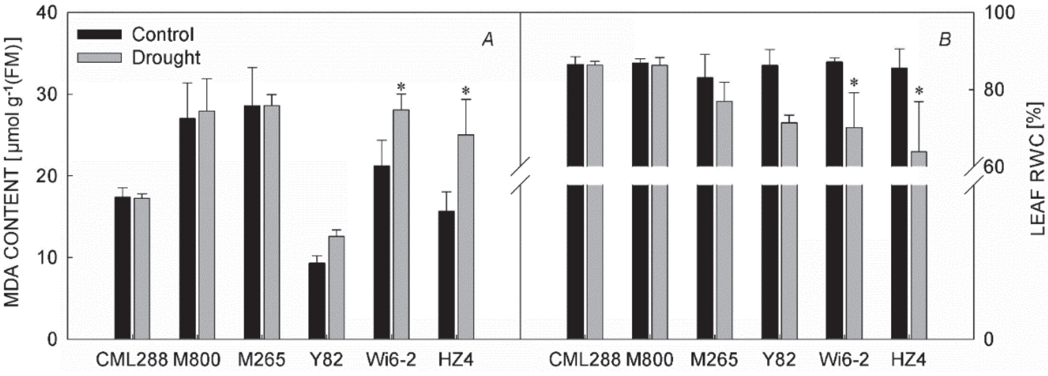

Fig. 2. Effects of drought stress on MDA content (*A*) and relative water content (RWC) (*B*) of leaves in maize inbred lines. Bars represent standard errors of triplicate experiments. *Different letters* indicate significant differences at $p<0.05$; * or ** indicate significant differences between control and drought stress at $p<0.05$ and $p<0.01$, respectively (one-way *ANOVA*).

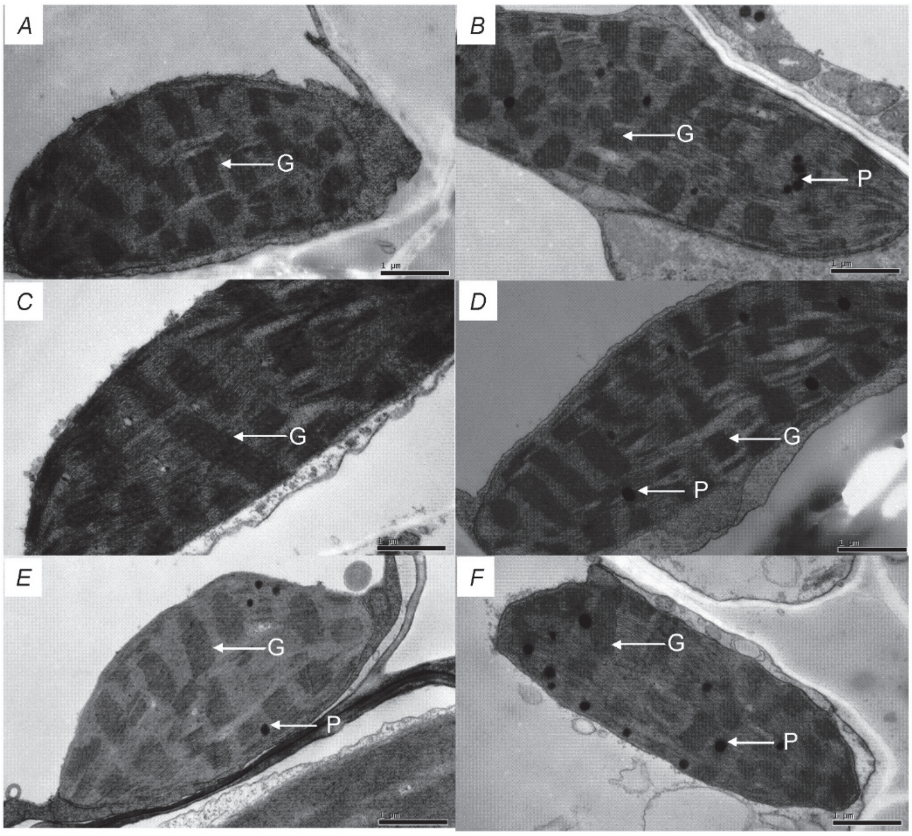

Fig. 3. Transmission electron micrographs of leaf chloroplast ultrastructure from three maize inbred lines (CML288, M800, M265): (*A*) CML288 seedlings grown under normal condition; (*B*) CML288 seedlings grown under drought stress for 2 d; (*C*) M800 seedlings grown under normal conditions; (*D*) M800 seedlings grown under drought stress for 2 d; (*E*) M265 seedlings grown under normal conditions; (*F*) M265 seedlings grown under drought stress for 2 d; Bars = 1 μm; G – granal thylakoids; P – plastoglobuli; SG – starch grains; V – vesicle. At least fifteen chloroplasts from each treatment were examined.

parameters showed that lines CML288 and M800 were the most tolerant to drought stress, M265 was the moderately tolerant line, and Wi6-2 and HZ4 were the most sensitive to drought stress, followed by Y82 which was also somewhat sensitive to drought.

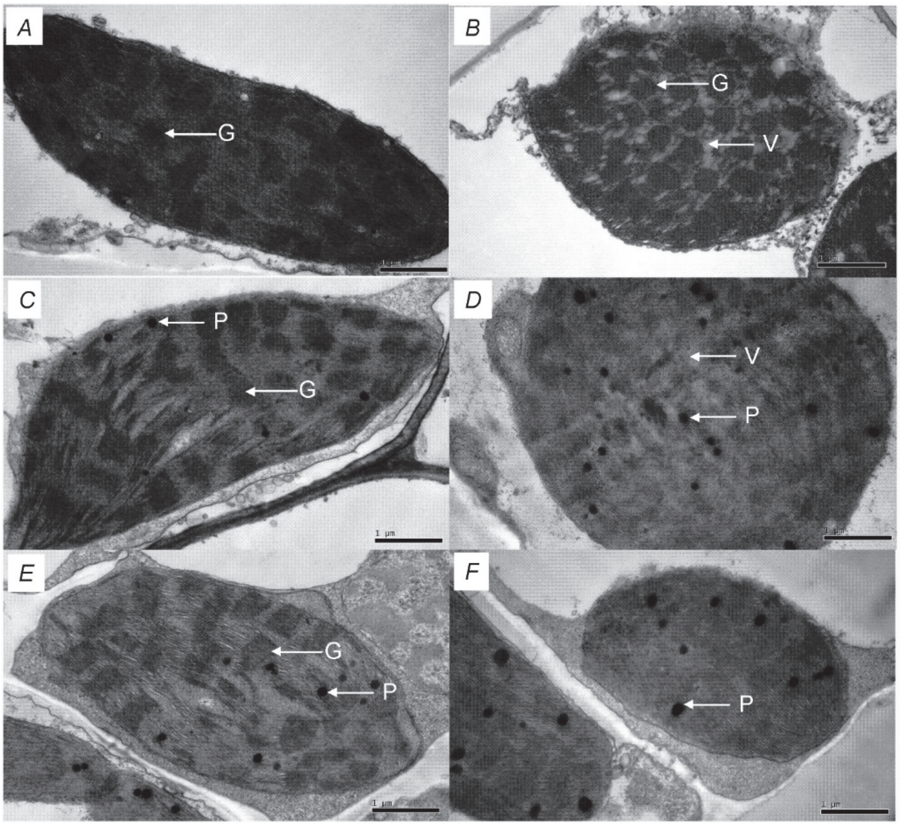

Fig. 4. Transmission electron micrographs of leaf chloroplast ultrastructure from three maize inbred lines (Y82, Wi6-2, HZ4): (*A*) Y82 seedlings grown under normal conditions; (*B*) Y82 seedlings grown under drought stress for 2 d; (*C*) Wi6-2 seedlings grown under normal conditions; (*D*) Wi6-2 seedlings grown under drought stress for 2 d; (*E*) HZ4 seedlings grown under normal conditions; (*F*) HZ4 seedlings grown under drought stress for 2 d. Bars = 1 μm; G – granal thylakoids; P – plastoglobuli; SG – starch grains; V – vesicle. At least fifteen chloroplasts from each treatment were examined.

Table 1. Morphometric analysis of ultrathin sections of mesophyll cells from six inbred lines of maize, grown on Hoagland nutrient solution without PEG (control) and with 20% PEG 6000 (–0.6 MPa, drought stress). Data were obtained by analyzing on average 20 micrographs per inbred line. * – significant difference for each trait within each line between contol and drought stress at $p<0.05$.

Maize inbred lines	Average chloroplast length [μm]		Average chloroplast width [μm]		Length-to-width ratio of chloroplasts		Cell area occupied by chloroplasts [%]	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought
CML288	4.40 ± 1.66	3.41 ± 0.66	1.86 ± 0.36	1.54 ± 0.32	2.37	2.21	40.3 ± 7.3	39.8 ± 12.6
M800	3.65 ± 0.87	3.51 ± 1.20	1.38 ± 0.15	1.50 ± 0.54	2.54	2.42	35.3 ± 10.2	34.3 ± 0.9
M265	3.53 ± 0.80	3.20 ± 0.41	1.30 ± 0.38	1.37 ± 0.30	2.70	2.33	30.6 ± 8.3	28.7 ± 7.3
Y82	3.16 ± 0.88	2.04 ± 0.51*	1.34 ± 0.40	1.11 ± 0.40	2.36	1.84*	19.8 ± 3.9	15.3 ± 0.2*
Wi6-2	3.20 ± 0.22	3.41 ± 0.63	1.53 ± 0.27	2.49 ± 0.67*	2.09	1.37*	28.3 ± 9.7	20.1 ± 9.3*
HZ4	3.37 ± 0.93	3.35 ± 0.50	1.39 ± 0.53	1.94 ± 0.50*	2.41	1.73*	24.5 ± 6.4	17.3 ± 3.1*

Ultrastructural and morphometric changes in chloroplasts: Chloroplasts of control plants occupied 20–40 % of the cell volume; the average chloroplast length in all lines ranged from 3.2–4.5 μm and the length-to-width ratios of chloroplasts were about 2.0 with no significant differences between lines (Table 1). The shapes of chloroplasts were lens-like and oblong, with a typical arrangement of granal and stromal thylakoids (Figs. 3, 4). Leaf mesophyll cells were characterized by well differentiated chloroplasts containing fully developed grana with numerous stacked thylakoids and well-developed stroma lamellae with a small number of plastoglobuli (Figs. 3, 4).

Following drought stress, there was no significant change in the cell area occupied by chloroplasts and in the size and length-to-width ratio of chloroplasts (Table 1) in the drought-resistant lines; the typical fine structure of chloroplasts was not disintegrated, and no intrathylakoid spaces appeared, although small plastoglobuli showed a high electron density (Fig. 3). However, in drought-sensitive lines, a significant reduction in the length-to-width ratios of chloroplasts and the cell area occupied by chloroplasts was observed (Table 1); the typical fine structure of chloroplasts and mitochondrial cristae were disintegrated, and plastoglobuli also showed a high electron density (Fig. 4). It should be emphasized that the drought-sensitive lines exhibited the highest degree of disorganization in fine thylakoid structure due to swelling of thylakoids and disappearance of grana; this changed their shape to round or oval, and the intra-thylakoid space therefore increased.

Discussion

In the future, the current trend for more severe and frequent drought periods will be exacerbated by further global warming, deforestation, and urbanization. Thus, water deficit is a global issue to ensure survival of agricultural crops and sustainable food production (Gleeson *et al.* 2012). Water stress results in water loss within plant tissues (Deeba *et al.* 2012). The intensity of growth inhibition following drought stress depends on the growth stage of the crop, the severity of drought, and drought tolerance of the plant genotype, with young seedlings being the most sensitive to water scarcity (Fulda *et al.* 2011). In our study, LMDI (about 0.5) can be used to classify the inbred lines as drought-sensitive or drought-resistant, because of leaf sensitivity to water deficit – this is in agreement with previous studies (Hunt and Rock 1989, Pereyra-Irujo *et al.* 2008, Xie *et al.* 2013). Plant height in the drought-sensitive lines was reduced to different degrees by water deficit (Fig. 1); this growth inhibition is well known and can support survival under drought. The above changes are typical of drought-stressed plants, where such developmental changes are known to be induced by an increase of lipid peroxidation (Miller *et al.* 2010).

Generally, drought-induced lipid peroxidation results in a massive reorganization of leaf chloroplast ultrastructure and Chl content (Anjum *et al.* 2011), which is considered to be a good indicator of plant status under conditions of stress (Xu *et al.* 2009). In this study, the impairment of leaf structure occurred due to drought stress mainly in the drought-sensitive lines, where leaf cells exhibited an obvious breakdown of the chloroplast membrane system, observed as barely distinguishable grana and a large number of small plastoglobuli. Other researchers have also found that upon drought treatment, granal thylakoids degrade concomitantly with the increase in the plastoglobuli number and size (Vassileva *et al.* 2012, Xu *et al.* 2009), which was consistent with our results. However, chloroplasts in the drought-tolerant lines remained more intact during the drought period. As the light reactions of photosynthesis occur in the grana, a close relationship is expected between membrane integrity and photosynthetic activity (Bonini *et al.* 2007, Zheng *et al.* 2011). It should also be noted that drought exposure changed the shape of many chloroplasts from lenticular to round or oval, suggesting that an altered function could also occur. To convert visible structural changes into numerical data, the length and width of chloroplasts were measured and the chloroplast shape was estimated for each line. The PEG-induced drought provoked a significant reduction in the length-to-width ratios mainly in chloroplasts of the drought-sensitive lines. Zellnig *et al.* (2004) observed that stress-impaired growth in leaves of drought-sensitive spinach lines was related to the reduced length and abnormal shape of chloroplasts, which is regarded as a sign of enhanced water loss in leaves. Thus, the evaluation of drought-induced changes in the chloroplasts ultrastructure of leaves could be a useful marker for discrimination of drought tolerant *vs.* susceptible inbred lines of maize.

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