Systemic Regulation of Leaf Anatomical Structure, Photosynthetic Performance, and High-Light Tolerance in Sorghum\(^1\)\(^{[C]}\)

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Leaf anatomy of C3 plants is mainly regulated by a systemic irradiance signal. Since the anatomical features of C4 plants are different from that of C3 plants, we investigated whether the systemic irradiance signal regulates leaf anatomical structure and photosynthetic performance in sorghum (Sorghum bicolor), a C4 plant. Compared with growth under ambient conditions (A), no significant changes in anatomical structure were observed in newly developed leaves by shading young leaves alone (YS). Shading mature leaves (MS) or whole plants (S), on the other hand, caused shade-leaf anatomy in newly developed leaves. By contrast, chloroplast ultrastructure in developing leaves depended only on their local light conditions. Functionally, shading young leaves alone had little effect on their net photosynthetic capacity and stomatal conductance, but shading mature leaves or whole plants significantly decreased these two parameters in newly developed leaves. Specifically, the net photosynthetic rate in newly developed leaves exhibited a positive linear correlation with that of mature leaves, as did stomatal conductance. In MS and S treatments, newly developed leaves exhibited severe photoinhibition under high light. By contrast, newly developed leaves in A and YS treatments were more resistant to high light relative to those in MS- and S-treated seedlings. We suggest that (1) leaf anatomical structure, photosynthetic capacity, and high-light tolerance in newly developed sorghum leaves were regulated by a systemic irradiance signal from mature leaves; and (2) chloroplast ultrastructure only weakly influenced the development of photosynthetic capacity and high-light tolerance. The potential significance of the regulation by a systemic irradiance signal is discussed.

Light is one of the most important environmental factors that regulate the development of the photosynthetic apparatus in higher plants. In high or low light, plants develop sun or shade leaves, respectively (Boardman, 1977; Anderson, 1986). The differences between typical sun and shade leaves in relation to anatomy and physiology have been extensively studied. Generally, leaves developed under high light are thicker and smaller, with more developed palisade tissue and higher stomatal density on both adaxial and abaxial surfaces compared with shade leaves (Anderson and Osmond, 1987; Murchie and Horton, 1997; Chen et al., 2002). Similarly, chloroplast ultrastructure also changes with growth irradiance. Sun-type chloroplasts have less appression of thylakoid membranes, while shade-type chloroplasts have more appressed thylakoid membranes (Anderson, 1986; Anderson and Osmond, 1987; Terashima and Hikosaka, 1995; Chow et al., 2005; Anderson et al., 2008). Functionally, sun leaves have higher photosynthetic capacity, higher amounts of ribulose bisphosphate carboxylase/oxygenase, and of electron transfer carriers than shade leaves on a leaf area basis. Accordingly, sun leaves have a strong high-light tolerance owing to high rates of carbon assimilation and enhanced ability to dissipate excess light energy, whereas shade leaves exhibit an increased susceptibility to damage by high light (Demmig-Adams and Adams, 1992; Osmond and Förster, 2006).

Previous investigations focused on leaf structure and function in plants grown fully under high or low light. However, in practice, close planting of crops always leads to a weak-light environment around the lower mature leaves, while the upper developing leaves are exposed to high light. Karpinski et al. (1999) demonstrated that partial exposure of low-light-adapted Arabidopsis (Arabidopsis thaliana) plants to excess light resulted in a systemic acclimation to excess excitation energy and to consequent photooxidative stress in untreated leaves kept in low light. Since then, some studies have reported that stomatal density, leaf thickness, and the development of stomatal and palisade tissue in newly developed leaves are

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independent of their local irradiance in Arabidopsis, poplar (Populus spp.), and tobacco (Nicotiana tabacum), but instead depend on the light environment of mature leaves (Lake et al., 2001; Thomas et al., 2004; Coupe et al., 2006; Miyazawa et al., 2006). This long-distance signal from mature to developing leaves is defined as a systemic irradiance signal. However, all these studies were conducted in C3 plants but, to our knowledge, no attention has been paid to C4 plants.

The anatomical features of C4 plants are largely different from those of C3 plants. For most C3 plants, the mesophyll differentiates into the palisade layer (lying beneath the adaxial epidermis) and the spongy layer (lying above the abaxial epidermis), while isobilateral leaves of C4 plants have palisade layers on both sides of leaves, or only have parenchyma cells, without differentiation into palisade and spongy tissue. Most importantly, C4 leaves are characterized by Kranz-type anatomy, in which the vascular bundle is surrounded by organelle-rich bundle sheath cells, which are in turn surrounded by radially arranged mesophyll cells. Functionally, in C4 photosynthesis, atmospheric CO$_2$ is initially fixed in the mesophyll cells, followed by decarboxylation and refixation of CO$_2$ in the bundle sheath cells (Sage, 2002; Majeran and van Wijk, 2009). Given the differences in anatomical structure between C4 plants and C3 plants, we wondered whether the regulation of the anatomical structure of developing leaves by a systemic signal in a C4 plant occurs in the same way as in C3 plants. In addition, although leaf anatomical structure may be markedly regulated by systemic signaling, the ultrastructure of chloroplasts depends on their local light environment during leaf development (Yano and Terashima, 2001). Since both leaf anatomy and chloroplast ultrastructure provide a structural framework for photosynthetic performance, in this study we also investigated whether photosynthetic capacity and tolerance of high light in developing leaves are determined by the systemic irradiance signal from mature leaves.

Sorghum, a typical C4 plant with isobilateral leaves, is one of the most important energy crops in the world with a very high yield of biomass. Using sorghum seedlings, we addressed the following questions by analyzing leaf anatomy, chloroplast ultrastructure, gas exchange, and chlorophyll (Chl) fluorescence: (1) how the systemic irradiance signal influences leaf anatomy in a typical C4 plant, and (2) whether the systemic irradiance signal regulates photosynthetic capacity and high-light tolerance. This study will give a new perspective for understanding both leaf development and the relationship between the photosynthetic apparatus in different locations within the plant.

RESULTS

Changes in Stomatal Density

The stomatal density in newly developed leaves on sorghum plants after the YS treatment (only young leaves shaded) showed no significant changes compared with the A treatment (plants grown in ambient conditions without shading); in contrast, shading mature leaves (MS) or whole plants (S) caused a marked reduction in stomatal density of newly developed leaves in the MS or S treatment (Fig. 1, A and B). In the MS treatment, shading mature leaves decreased the stomatal density by 30% on the adaxial surface and 15% on the abaxial surface in newly developed leaves,
compared with the respective A treatment. These results suggest that stomatal density in young leaves is mainly controlled by the light environment of mature leaves. Interestingly, in newly developed leaves, the stomatal density on the adaxial surface was more influenced by the light environment of mature leaves than was that on the abaxial surface (Fig. 1).

Changes in Leaf Anatomical Structure

The leaf anatomical features of typical C4 plants, with no differentiation into palisade tissue and spongy tissue, are very different from those of C3 plants with dorsiventral leaves. The effects of shading treatments on cross sections of newly developed sorghum leaves are shown visually in Figure 2. Newly developed leaves after MS and S treatments were thinner than those after A and YS treatments (Fig. 3A), indicating that the thickness of newly developed leaves was determined by the light environment of mature leaves. However, the mesophyll thickness of adaxial and abaxial sides responded differentially (Fig. 3, C and D). The adaxial mesophyll thickness decreased by 16% and 23% in MS and S treatments compared with that in A treatment, respectively; by contrast, the decrease of mesophyll thickness on the abaxial side was less than 10% in either treatment. This observation implies that the adaxial mesophyll thickness in newly developed leaves was more sensitive than the abaxial mesophyll thickness in response to shading of mature leaves.

Usually, C4 leaves are characterized by Kranz-type anatomy, in which the vascular bundle is surrounded by organelle-rich bundle sheath cells, and this tissue layer is further surrounded by radially arranged mesophyll cells. In C4 photosynthesis, atmospheric CO₂ is initially fixed in the mesophyll cells, and then delivered to the bundle sheath cells. It is in the bundle sheath cells that decarboxylation and refixation of CO₂ occur (Sage, 2002; Majeran and van Wijk, 2009). Apparently, metabolite transfer between the bundle sheath and mesophyll cells is a central factor for the regulation of C4 photosynthesis (von Caemmerer and Furbank, 1999). The contact area between bundle sheath and mesophyll cells, indicated by $S_b$, is related to the ability to transfer the metabolites that ensure the efficient operation of C4 photosynthesis (Soares-Cordeiro et al., 2009). A higher value of $S_b$ indicates a more rapid metabolite transfer between bundle sheath and mesophyll (Sowinski et al., 2008; Soares-Cordeiro et al., 2009). Therefore, the contact area between bundle sheath and mesophyll cells was determined. We observed that shading mature leaves caused a distinct decline in $S_b$ in newly developed leaves in the MS and S treatments (Fig. 3B); by contrast, little or no change was observed in the YS treatment, suggesting that the surface area of contact between bundle sheath and

Figure 3. Effects of shading treatments on leaf thickness (A), contact area of bundle sheath cells ($S_b$; B), adaxial (C), and abaxial (D) mesophyll thickness in newly developed leaves. Data are means ± se of six replicates. Note that the y axis on some sections does not begin at zero.
mesophyll cells is regulated by the light environment of mature leaves.

Changes in Chloroplast Ultrastructure

Changes in the ultrastructure of chloroplasts are shown visually in Figures 4 and 5. Newly developed leaves after A and MS treatments had thinner granal stacks compared with YS and S treatments (Fig. 5A). To further quantify the degree of thylakoid stacking, the ratio of the cross-sectional area of all appressed thylakoids \((S_g)\) to that of the chloroplasts \((S_c)\) was determined, this ratio reflecting the extent to which the chloroplast volume was occupied by appressed thylakoids. Shading developing leaves, but not mature leaves, increased \(S_g/S_c\) in newly developed leaves (Fig. 5B). These data indicate that the chloroplast ultrastructure in developing leaves depended on their local light condition and was relatively independent of the light environment of mature leaves.

Changes in Gas Exchange

The net photosynthetic rates \((P_n)\) of mature and newly developed leaves at irradiances 800 and 1,200 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) are shown in Figure 6, A and C, respectively. There was little difference between \(P_n\) of mature leaves in the A and YS treatments (Fig. 6A). By contrast, \(P_n\) in mature leaves with MS and S treatments decreased significantly compared with those in A and YS treatments under both 800 and 1,200 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (Fig. 6A). When subjected to 1,200 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\), the net photosynthetic rate of mature leaves in MS and S treatments were 20.6 and 21.1 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), respectively, which were 35% and 33.6% lower than those in A treatments under 1,200 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (Fig. 6A). For newly developed leaves, the net photosynthetic rates in seedlings after MS and S treatments were lower than those after A and YS treatments (Fig. 6C). Stomatal conductance in both mature leaves and newly developed leaves showed similar trends to net photosynthetic rates in all treatments (Fig. 6, B and D). These results suggest that the light environment of mature leaves had a strong impact on the net photosynthetic rate and stomatal conductance not only in themselves but also in developing leaves.

Changes in Chl a Fluorescence

As shown in Figure 7, the initial Chl fluorescence yield \((F_o)\), maximum Chl fluorescence yield \((F_m)\), or maximum quantum yield of PSII photochemistry \((F_v/F_m)\) were each similar among all treatments at 6 AM (Fig. 7), indicating that all shading treatments did not bring about significant differences in the predawn photochemical efficiency of PSII, whether in mature or newly developed leaves. During early afternoon (2 pm), however, an obvious increase in \(F_o\) together with a significant decline in \(F_m\) occurred in shaded mature leaves with MS and S treatments after exposure of horizontally held leaves to high irradiance, while the values of \(F_o\) and \(F_m\) in mature leaves with A and YS treatments remained relatively constant (Fig. 7, A and B). Consequently, \(F_v/F_m\) at early afternoon decreased significantly in shaded mature leaves in MS and S treatments but did not decrease significantly in exposed mature leaves in A and YS treatments (Fig. 7C). In newly developed leaves, all these parameters showed similar trends to those of the mature leaves (Fig. 7, D–F). Therefore, shading mature leaves induced an increased susceptibility of PSII to photo-
inhibition upon exposure to high light, not only in themselves but also in newly developed leaves.

DISCUSSION

Systemic Regulation of Leaf Morphology and Anatomy

In most previous investigations on light acclimation, the regulation of photosynthesis in a single leaf has been extensively studied. To our knowledge, no attention has been paid to the impact of shading a single leaf of a C4 plant on the photosynthetic apparatus and performance of leaves elsewhere on the same plant. In this study, we demonstrated that the anatomy of newly developed leaves on a typical C4 plant changed significantly after shading mature leaves (in the MS treatment), as if the young leaves had developed in weak light though exposed to high irradiance. By contrast, shading developing leaves alone caused little change in the anatomical characteristics of newly developed leaves themselves (in the YS treatment). Our results demonstrate that in sorghum seedlings, it is the light environment of the mature leaves, not the local light environment of developing leaves, which controls the development of anatomical structure in newly developed leaves. Therefore, we suggest that there is a systemic irradiance signal from mature leaves to developing leaves in C4 plants, as has been suggested for some C3 plants (Lake et al., 2001; Thomas et al., 2004; Coupe et al., 2006; Miyazawa et al., 2006).

Specifically, we observed a significant decrease in stomatal density (Fig. 1) and in leaf thickness (Fig. 3A) of newly developed leaves due to the systemic irradiance signal from mature leaves. The systemic irradiance signal also resulted in a decrease in the contact area between bundle sheath and mesophyll cells in newly developed leaves in the MS treatment (Fig. 3B). Accordingly, we suggest that changes in stomatal density, leaf thickness, and the contact area between bundle sheath and mesophyll are the main targets of systemic regulation of leaf morphology and anatomy in sorghum seedlings. Moreover, the regulation of the morphology and anatomy of isobilateral leaves of sorghum by the systemic irradiance signal was asymmetrical: The adaxial stomatal density and mesophyll thickness in newly developed leaves, compared with the abaxial stomatal density and mesophyll thickness, were much more sensitive to shading of mature leaves (Fig. 3, C and D). Long et al. (1989) demonstrated that there is a physical CO2 diffusion barrier between adaxial and abaxial sides of C4 isobilateral leaves; therefore, the adaxial and abaxial sides of C4 isobilateral leaves can be viewed as separate compartments in terms of CO2 diffusion and assimilation. The two separate compartment system is useful not only in the optimization of whole-leaf photosynthesis, but also allows the separation in the signaling of stress and in the effects of stress factors (Long et al., 1989; Soares-
Cordeiro et al., 2009). In our study, it was the systemic irradiance signal from mature leaves that played a key role in the regulation of morphology and anatomy in newly developed leaves. Probably, the transportation and distribution of systemic irradiance signal molecules coming from mature leaves may be asymmetrical between the adaxial and abaxial sides of leaf, or the two sides of a leaf have different sensitivity to the systemic irradiance signal. The asymmetrical regulation of morphology and anatomy in newly developed C4 leaves, observed in our investigation, and its detailed mechanisms need further investigation.

Besides the anatomical differences, sun and shade leaves differ in their chloroplast ultrastructure. The ultrastructure of chloroplasts (Figs. 4 and 5) in our study responded only to the local light environment of the developing leaf, not a systemic irradiance signal; that is, the chloroplasts differentiated into sun- or shade-type organelles according to the local light environment. Therefore, our data provide clear evidence that sun- or shade-type chloroplast development is independent of the anatomical differentiation of the tissue in the developing leaves. Our conclusion on chloroplast ultrastructural changes obtained with sorghum seedlings is consistent with that obtained with the C3 plant Chenopodium album (Yano and Terashima, 2001). Of course, the development of chloroplasts may influence the development of the leaf under extreme conditions, as reported previously (Chatterjee et al., 1996; Keddie et al., 1996). However, this phenomenon was not observed in this study.

**Systemic Regulation of Photosynthetic Capacity and High-Light Tolerance**

In this study, shading developing leaves alone had little effect on their photosynthetic capacity and stomatal conductance in the YS treatment, while the photosynthetic capacity and stomatal conductance of newly developed leaves in MS and S treatments declined with the decrease in net photosynthetic rate and stomatal conductance of mature leaves. Significantly, we observed a positive linear correlation between a functional parameter ($P_n$ or stomatal conductance $[G_s]$) in newly developed leaves and that in mature leaves (Fig. 8). Therefore, we suggest that the development of photosynthetic capacity and stomatal conductance in developing leaves is also regulated by systemic irradiance signal from mature leaves.

In our investigation, photoinactivation of PSII in both mature and newly developed leaves in MS and S treatments was also clearly exacerbated following exposure to high irradiance (Fig. 7, C and F), owing to their depressed photosynthetic capacity. There are two mechanisms that are primarily responsible for initiating the photoinactivation of PSII, one of which operates when excess light energy is not utilized by photosynthesis (Oguchi et al., 2009). The lower the photosynthetic capacity, as was the case in the MS and S treatments, the greater was the excess energy, consistent with the exacerbation of photoinactivation of
PSII. On the other hand, $F_v/F_m$ in newly developed leaves in the YS treatment was hardly affected by exposure to high light; this is consistent with there being little or no effect of the YS treatment on $P_n$ (Fig. 6C). Therefore, we conclude that not only photosynthetic capacity, but also high-light tolerance in newly developed leaves are determined by a systemic irradiance signal from mature leaves.

Leaf morphological characteristics and anatomical structure play a crucial role in the regulation of photosynthetic performance, providing a structural framework for the diffusion of gases and the optimization of photosynthetic activity (Terashima and Inoue, 1985). For developing leaves, stomatal density, leaf thickness, and $S_n$ in MS treatment were all regulated by systemic irradiance signal in this study. Therefore, we deduce that the changes in morphological characteristics and anatomical structure of newly developed leaves in C4 plant may be at least partially responsible for the alteration of photosynthetic capacity and high-light tolerance. On the other hand, for fully expanded leaves under weak light, the role of leaf anatomy in the acclimation of photosynthesis to high light is very limited (Oren et al., 1986; Oguchi et al., 2003). Accordingly, during shading treatment, decreased photosynthetic capacity in mature leaves in MS and S treatments probably resulted from physiological acclimation to low light, rather than leaf morphology and anatomy that are fixed in mature leaves.

On an ultrastructural level, changes also occurred in chloroplasts. The membranes in chloroplasts of higher plants are differentiated into granal and stromal thylakoids: Shade-type or sun-type chloroplasts are formed according to growth irradiance, such that an increase in growth irradiance decreases granal stacking in chloroplasts (Anderson, 1986; Anderson and Osmond, 1987; Terashima and Hikosaka, 1995; Chow et al., 2005; Anderson et al., 2008). Recently, it was hypothesized that the functions of granal stacking include a potential increase of photosynthetic capacity. This is because, all else being equal, better formation of grana should allow more space for free diffusion of large enzyme complexes of the Calvin-Benson cycle in a very crowded stroma (Chow et al., 2005; Anderson et al., 2008). That is, the formation of large grana should not diminish, but probably enhance, photosynthetic capacity, all else being equal. Interestingly, in the YS treatment, we observed that newly developed leaves were like sun leaves with shade-type chloroplast ultrastructure, exhibiting high net photosynthetic capacity and strong tolerance of high light but possessing large granal stacks. It appears from this observation that, indeed, large grana did not diminish photosynthetic capacity. In the MS treatment, newly developed leaves were like shade leaves exhibiting a low photosynthetic capacity and an increased susceptibility to high-light stress, but possessing sun-type chloroplasts with small granal stacks. It appears from this observation that poor granal formation did not aid in increasing photosynthetic capacity. Together, the data suggest that the ultrastructure of chloroplasts or granal stacking observed in the YS and MS treatments was consistent with photosynthetic capacity and high-light tolerance. However, in the S treatment, although the grana of newly developed leaves were large, the photosynthetic capacity was small. Presumably, other more dominant factors in the S treatment overrode any positive granal effect on photosynthetic capacity.

Our data demonstrated that the weak-light environment around mature leaves is adverse to the development of photosynthetic capacity and high-light tolerance in developing leaves, owing to the existence of a systemic irradiance signal in plants. Therefore, achieving an appropriate planting density and decreasing mutual shading among adjacent mature leaves would enhance the photosynthetic capability in both mature leaves and developing leaves and consequently their resistance to strong light.

**CONCLUSION**

In a C4 plant, we demonstrated that anatomical structure, photosynthetic capacity, and high-light tolerance in newly developed leaves were regulated by a systemic irradiance signal originating in mature leaves, just as in C3 plants. During leaf development, chloroplast ultrastructure played only a weak role in the regulation of photosynthetic capacity and high-light tolerance. This study could provide a new perspective for understanding the relationship between leaf development and photosynthetic performance.

**MATERIALS AND METHODS**

**Plant Growth**

Sorghum (Sorghum bicolor L. cv ‘Liaoza 10’) seeds were imbibed on wet paper for 1 d. The germinated seeds were sown in 30 × 20 cm containers filled with vermiculite. Plants were watered every 2nd d. One week later, seedlings were transplanted into pots (15 cm in diameter, 20 cm in height) containing Hoagland solution and grown in water culture in a greenhouse with a maximum irradiance of 1,217 ± 26 μmol m$^{-2}$ s$^{-1}$ and a day/night temperature of 35°C/22°C. Relative humidity was 40% to 60%. The nutrient solution contained 5 m M KNO$_3$, 1 m M KH$_2$PO$_4$, 1 m M CaCl$_2$, 5 m M Ca(NO$_3$)$_2$, 2 m M MgSO$_4$, 0.08 m M FeEDTA, plus trace elements (0.05 m M H$_3$BO$_4$, 0.009 m M MnCl$_2$, 4 H$_2$O, 0.0008 m M ZnSO$_4$, 7H$_2$O, 0.0004 m M CuSO$_4$, 5H$_2$O, 0.0039 m M H$_2$MoO$_4$, 4H$_2$O), pH 5.5. The seedlings, with the developing true leaf number 6 about 5 cm in length (soon after it had emerged), were then divided into four groups for different shading treatments, and grown for a further 14-d period. During the experiment, the Hoagland solution was topped up every 3 d.

**Shading Treatments**

Four treatments were used: A, YS, MS, and S. The irradiance at the exposed leaves was about 1,200 μmol m$^{-2}$ s$^{-1}$ at noon; target leaves or seedlings were shaded by a piece of nylon net (Fig. 9), the maximum attenuated irradiance being about 300 μmol m$^{-2}$ s$^{-1}$. Two weeks later, when the true leaf number 6 became fully expanded, the middle section of true leaf number 4 (mature leaves) and number 6 (newly developed leaves) were used for all measurements in this experiment. Every treatment had at least six replicates.

**Measurement of Gas Exchange**

Gas-exchange measurements were carried out using a portable gas-exchange system (CIRAS-2, PP-Systems) with ambient CO$_2$ concentration.
Measurement of Chlorophyll a Fluorescence

Chlorophyll a fluorescence was measured with a handy plant efficiency analyzer (Hansatech). Fully dark-adapted seedlings (12 h) were used to determine the F<sub>R</sub>/F<sub>M</sub> at 6 AM. After the initial F<sub>R</sub> was measured in modulated measuring light of negligible irradiance, a 1-s pulse of saturating red light (3,500 μmol m<sup>−2</sup> s<sup>−1</sup>) was applied to obtain the F<sub>R</sub> and F<sub>M</sub> was calculated as (F<sub>M</sub> – F<sub>R</sub>)/(F<sub>M</sub> – F<sub>L</sub>) where F<sub>L</sub> is the variable chlorophyll fluorescence yield (Genty et al., 1989; Bilger and Björkman, 1990). Plants were then placed under natural irradiance (1,400–1,600 μmol m<sup>−2</sup> s<sup>−1</sup>) with leaves stretched horizontally from 8 AM to 2 PS for 6 h. F<sub>R</sub>/F<sub>M</sub> at 2 PS was measured after dark adaptation for 10 min.

Counting of Stomata

Stomatal density was determined following the method of Coupe et al. (2006). Once the developing leaves had become fully expanded, nail polish was applied to the leaf surface to obtain a replica of the leaf. The replicas were observed under a light microscope (Nikon-E800) and a digital camera was used to photograph the replicas. The number of stomata was counted in six fields of view from the six marked leaves of six individual replicas were observed under a light microscope (Nikon-E800) and a digital camera (BH-2, Olympus). Leaf thickness and mesophyll thickness were obtained using Photoshop software and six different positions were measured (Leica Ultracut R) and stained with uranyl acetate and lead citrate double (2006). Once the developing leaves had become fully expanded, nail polish was applied to the leaf surface to obtain a replica of the leaf. The replicas were observed under a light microscope (Nikon-E800) and a digital camera was used to photograph the replicas. The number of stomata was counted in six fields of view from the six marked leaves of six individual plants for each treatment.

Measurement of Leaf Thickness, Mesophyll Thickness, and Contact Area of Bundle Sheath Cells

Leaf segments (2 × 2 mm) without major veins were cut from the basal part of the leaf lamina with a razor blade. The segments were fixed in a solution containing 5% formalin, 5% acetic acid, and 90% ethanol at 4°C. The fixed segments were dehydrated in a graded series of ethanol solutions and embedded in Spurr’s resin (Ladd). Light microscopy was carried out with a μm-thick transverse sections of the leaf cut with a glass knife on an ultramicrotome (Leica Ultracut R) and stained with 0.5% toluidine blue. Light micrographs were taken with a digital camera (BH-2, Olympus). Leaf thickness and mesophyll thickness were obtained using Photoshop software and six different positions were measured in each segment. The adaxial and abaxial mesophyll thickness was measured separately relative to the middle of the bundle sheath, which in general corresponded to the middle of the leaf. In Figure 10, the measurement of the adaxial and abaxial mesophyll tissues is shown in a cross section micrograph, and calculation of the contact area of bundle sheath cells (μm<sup>2</sup> μm<sup>−1</sup>) is explained in the legend.

Chloroplast Ultrastructure

Leaves were sampled within 2 h from the start of the light period. The segments (1 × 1 mm) were fixed at 4°C in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and then treated with 1% osmium tetroxide overnight at 4°C. The fixed segments were dehydrated in a graded acetone series and embedded in Spurr’s resin (Ladd). Transmission electron microscopy of chloroplast ultrastructure was done with 40-nm ultrathin sections cut with a diamond knife on the ultramicrotome (Leica Ultracut R) and stained with uranyl acetate and lead citrate double staining. Chloroplasts of the uppermost part of the leaf sections were viewed under an electron microscope (JEM 1230; JEOL) and electron micrographs were taken with a digital camera (BH-2, Olympus). Photographs of chloroplasts were analyzed for the calculation of the thickness of granal stacks and the ratio of the cross-sectional area of granal to that of chloroplasts (%). Statistical Analysis

Data were compared with the Duncan multiple comparison test using SPSS (version 13.0) at the level of 0.05. Correlations of linear regressions were calculated using SigmaPlot (version 10.0).

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