DOI: 10.32615/ps.2020.029 PHOTOSYNTHETICA 58 (3): 732-739, 2020

Light reacclimatization of lower leaves in C_4 maize canopies grown at two planting densities

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Abstract

C_4 plants have high photosynthetic capacity but are inefficient under low light. In a canopy, lower leaves developed under high light are progressively shaded. To elucidate how lower leaves in a C_4 canopy reacclimatize to low light, we investigated maize canopies differing in light environment grown at standard and low planting densities (SPD, LPD). Although upper leaves at SPD and both upper and lower leaves at LPD had light-response curves of photosynthesis of sun leaves, lower leaves at SPD had that of shade leaves. All leaves at both densities had anatomical framework of sun leaves, but the chloroplast content in mesophyll and bundle-sheath cells of lower leaves at SPD was greatly reduced to reacclimatize to low light. This study demonstrates that lower leaves at SPD reacclimatize to low light by adjusting their physiological and chloroplast traits while maintaining anatomical framework, whereas those at LPD behave as sun leaves.

Additional key words: chlorophyll content; chlorophyll fluorescence; leaf anatomy; leaf mass per area; leaf nitrogen content.

Introduction

Photosynthesis is one of the most critical physiological traits involved in crop productivity. However, some studies have reported that photosynthetic capacity is not necessarily correlated with crop yield, because many factors other than photosynthesis, such as the availability and uptake of water and nutrients, are also involved in crop yield (Sinclair *et al.* 2019 and references therein). On the other hand, several studies have shown that photosynthetic capacity is relevant to increasing crop yield (Jiang *et al.* 2003, Kromdijk *et al*. 2016). These contrasting conclusions may be caused by the fact that many of previous studies focused on photosynthetic performance only in individual leaves (Flood *et al.* 2011, Driever *et al.* 2014). An increase in crop yield *via* the improvement of photosynthetic traits would require the improvement of photosynthetic performance at the whole canopy level in the field (Evans 2013). Therefore, further studies to improve canopy photosynthesis of crops are required for future crop production.

Various environmental factors influence the canopy photosynthesis of crops. Among them, light is the most critical factor. Various traits of plants at leaf to canopy levels are affected by light intensity received during the growth period (Poorter *et al.* 2019). In a crop canopy, most upper leaves photosynthesize under full sunlight, whereas light intensity is gradually decreased with canopy depth owing to mutual shading. At the same time, the gradient of light quality (decreasing red to far-red ratio with canopy depth) is also caused by mutual shading. Therefore, both intensity and quality of light give rise to the gradation in leaf traits through a canopy (Pons 2016). As a result of mutual shading, lower leaves photosynthesize under limited light (Long *et al.* 2006). A substantial proportion of leaves in crops is distributed in the lower canopy (Monsi and Saeki 2005, Kromdijk *et al.* 2008). In addition, major crops recently tend to be planted at high densities. This would result in increasing shading portion of canopy (Richards 2000). Some studies have reported that a large proportion of CO_2 fixed by plants is derived from light-limited photosynthesis (Long 1993, Song *et al.* 2013). Therefore,

Received 16 June 2019, *accepted* 23 March 2020.
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Abbreviations: BS – bundle sheath; Chl – chlorophyll; DLI – daily light integral; F_m' and F_s – maximum and steady-state fluorescence yield of light-adapted leaves; IVD – interveinal distance; LCP – light-compensation point; LMA – leaf mass per area; LPD – low planting density; P_N – net photosynthetic rate; P_{2000} – P_N at PPFD = 2,000 μmol m^{-2} s^{-1}; R_D – dark respiration rate; SPD – standard planting density; Φ – photosynthetic quantum yield ; $Φ_{PSII}$ – quantum yield of photosystem II.
Acknowledgement: We are grateful to anonymous reviewers for their constructive comments on the manuscript and Prof. M. Kawasaki, Faculty of Agriculture, Hirosaki University, and the Shared Facility Center for Science and Technology, Hirosaki University for the use of an ultramicrotome. This study was supported by a Japan Society for the Promotion of Science KAKENHI grant (JP 16H04868) to O. Ueno.

the improvement of photosynthetic performance under limited and fluctuating light environments has increased importance in crop production (Yamori 2016, Slattery *et al.* 2018).

C_4 plants, including highly productive crops, such as maize and sorghum, have a CO_2-concentrating mechanism in their leaves (Hatch 1987, Leegood 2013). This mechanism is associated with structural differentiation of leaves: C_4 leaves generally have two types of photosynthetic cells, mesophyll and bundle-sheath (BS) cells (Hatch 1987, Leegood 2013). Atmospheric CO_2 is first fixed as C_4 acids by phospho*enol*pyruvate carboxylase in the mesophyll cells, and then the C_4 acids are decarboxylated in the BS cells. Released CO_2 is refixed by ribulose-1,5-bisphosphate carboxylase/oxygenase in the BS cells. This process increases the CO_2 concentration within the BS cells and thereby reduces photorespiration (Hatch 1987, Sage *et al*. 2012, Leegood 2013). Therefore, C_4 plants have higher photosynthetic capacity and productivity than C_3 plants under hot and high-light environments, where photorespiration is accelerated (Osmond *et al.* 1982, Brown 1999, Sage *et al*. 2012). On the other hand, C_4 plants are inefficient in shade (Osmond *et al.* 1982, Ehleringer and Monson 1993), because C_4 photosynthesis requires additional ATP to operate the CO_2-concentrating mechanism (Hatch 1987). This is evident at lower leaf temperatures, because C_4 plants show lower light-use efficiency than C_3 plants, since photorespiration of C_3 plants is reduced with decreasing temperature (Ehleringer and Monson 1993). In addition, the leakiness of CO_2 from BS cells reduces photosynthetic efficiency of C_4 plants under shade (Kromdijk *et al.* 2014). Although photosynthetic performance under limited light is unfavorable for C_4 plants, many leaves are distributed in the lower canopy (Kromdijk *et al.* 2008).

The light acclimatization of leaves has been well studied, especially in C_3 plants (reviewed in Björkman 1981, Givnish 1988, Lambers *et al.* 2008). Most studies have compared structural, biochemical, and physiological traits of typical sun and shade leaves generated under artificial, constant light. In the field, however, light intensity fluctuates widely. Therefore, to understand the effects of light environment on crop production in the field, we must study the light acclimatization of leaves in natural environments.

Furthermore, lower leaves in the crop canopy experience an inevitable light transition caused by canopy growth: these leaves develop under high light, but the light environment changes gradually from sunny to shady with canopy growth by mutual shading of leaves. Although such light transition occurs in almost all field crops, few studies have been carried out under field conditions with different daily light integrals (DLIs) and light fluctuations (Burkey and Wells 1991). Therefore, the light reacclimatization studies at canopy level will be required for our understanding of crop photosynthesis.

Recently, we have investigated the mechanism of reacclimatization to low light of maize leaves that had developed under high light in a shading experiment with pot-grown plants (Yabiku and Ueno 2019). The results showed that these leaves reacclimatize to low light by adjusting their biochemical traits and chloroplast contents to resemble shade leaves but maintain the anatomical framework of the sun leaves. However, it is still uncertain whether such light reacclimatization also occurs in the lower leaves of maize canopy grown in the field, since their light environment largely differs from the artificial light conditions in the shading experiment. Here, we compared photosynthetic and anatomical traits of lower leaves in maize canopies grown under standard and low planting densities to generate two different light environments. The results demonstrated that light reacclimatization similar to that found in our previous shading experiment occurs at the canopy level.

Materials and methods

Plant material: Maize plants (*Zea mays* L., cv. P1690, *Pioneer Ecoscience*, Tokyo, Japan) were grown in the field at Kyushu University, Fukuoka (33°35'N, 130°23'E) during late spring to mid-summer in 2016. P1690 was used in our previous pot study (Yabiku and Ueno 2019). First, the amounts of soluble N, P_2O_5, and K_2O in soil of the field were measured by use of a test stripe (*Midori-kun*, *Fujihira Industry*, Tokyo, Japan). The amount of basal fertilizer was determined from these data (data not shown). The field received 100 kg(N) ha^{-1} as ammonium sulfate, 50 kg(P_2O_5) ha^{-1} as single superphosphate, and 70 kg(K_2O) ha^{-1} as potassium chloride. The same amounts of N and K_2O were applied to plants at the fifth-leaf stage as top dressing. Two planting densities were set to generate different light environments in the lower canopy, using a row width of 60 cm and a plant spacing of either 24 cm (standard planting density, SPD: 6,900 plants ha^{-1}) or 60 cm (low planting density, LPD: 2,800 plants ha^{-1}). The former corresponded to planting density of cv. P1690 recommended by the manufacture (*Pioneer Ecoscience*). All plot sizes were 3.6 × 3.6 m, and plots were randomly arranged in the field. Seeds were sown on 17 May 2016. Data on solar radiation, temperature and precipitation during the period from May to September, 2016 in the experimental site (at 1 km^2 spatial resolution) were obtained from the Agro-Meteorological Grid Square Data of the National Agriculture and Food Research Organization (NARO), Japan (Ohno *et al.* 2016; Table 1S, *supplement*). The means of maximum and minimum temperature during the period (May to September) were 29.9 and 19.8°C, respectively. The DLI was calculated from global solar radiation, and the mean of DLI during the period was 44.9 mol(photon) m^{-2} d^{-1}. Plants were irrigated once a day but twice or three times on a fine day. Gas exchange was measured, and leaves were sampled for anatomical and physiological studies at the end of July, when the crop had formed a mature canopy at the maximum plant height (*ca.* 2.7 m). Two plants in the middle of each plot were measured and sampled, and values of each plot were represented by the mean of two plants. Values at each planting density are given as the means of three or four plots.

Canopy light intensity: The diurnal change of photosynthetic photon flux density (PPFD) was measured at the positions of the upper and lower leaves with a quantum sensor (*MIJ-14PAR Type 2*, *Environmental Measurement Japan*, Fukuoka, Japan) on fine days in the mid-summer. Fig. 1 showed diurnal course of PPFD on 29 July and 1 August, 2016 for SPD and LPD, respectively. At both densities, daily maximum PPFD in the upper leaves increased to > 2,000 μmol m^{-2} s^{-1} at midday, but that in the lower leaves was lower as a result of self-shading (Fig. 1). The lower leaves at SPD always received very low fluctuated light (PPFD < 500 μmol m^{-2} s^{-1}; Fig. 1*A*), whereas those at LPD received changing light intensity with several long sunflecks (from up to 1,750 μmol m^{-2} s^{-1} to < 400 μmol m^{-2} s^{-1}; Fig. 1*B*). DLI in the lower leaves was 19% of that in the upper leaves at SPD and 39% at LPD.

Gas exchange and chlorophyll (Chl) fluorescence: The light-response curve of photosynthesis in the third (upper) and ninth (lower) leaves numbered from the top was measured with a portable photosynthesis system (*LI-6400XT*, *LI-COR*, Lincoln, NE, USA) equipped with a fluorometer (*6400-40*, *LI-COR*), at a leaf temperature of 33°C, a relative humidity of 60%, and a CO_2 concentration of 400 μmol mol^{-1}. We chose the third leaves as representative of the upper leaves because a preliminary measurement showed that they had the highest net photosynthetic rate (P_N) among the upper leaves (data not shown). We chose the ninth leaves as representative of the lower leaves because they remained green and showed no signs of senescence. In the measurement of the light-response curve, the light intensity was increased stepwise from 0 to 20, 40, 60, 80, 100, 120, 140, 250, 500, 800; 1,100; 1,500; and 2,000 μmol m^{-2} s^{-1}. P_N was recorded when it stabilized at each light level after waiting for 10 to 20 min. The photosynthetic quantum yield (Φ) was calculated from the initial slope of the light-response curve. The dark respiration rate (R_D) and light-compensation point (LCP) were calculated from the intercepts of the tangent to the light-response curve at the limit of low light with the ordinate and abscissa, respectively (Lambers *et al*. 2008).

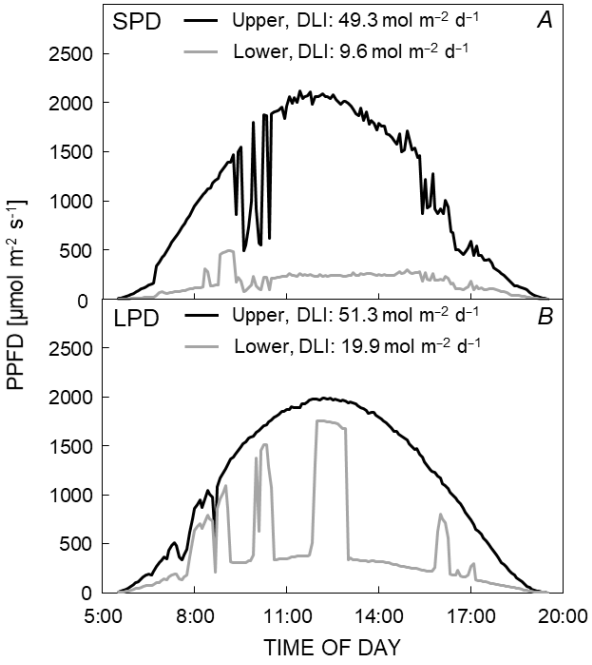

Fig. 1. Diurnal course of photosynthetic photon flux density (PPFD) in upper and lower leaves in maize canopies grown at (*A*) standard (SPD) and (*B*) low planting density (LPD). The measurements were made on 29 July, 2016 for SPD and 1 August, 2016 for LPD. DLI – daily light integral.

Chl fluorescence was measured simultaneously with the light-response curve with *6400-40* leaf chamber fluorometer. The steady-state yield of fluorescence (F_s) in response to modulated light [approx. 5 μmol(photon) m^{-2} s^{-1}] was recorded, and then the maximum fluorescence (F_m') was measured following a saturating light pulse [≥ 6,000 μmol(photon) m^{-2} s^{-1}, 0.8 s]. The quantum yield of PSII (Φ_{PSII}) was obtained as $\Phi_{PSII} = (F_m' - F_s)/F_m'$ (Genty *et al*. 1989).

Other physiological traits: Immediately after the gas-exchange and Chl fluorescence measurements, samples for measurement of Chl content, leaf mass per area (LMA), and N content were collected from the middle of the same leaves. Chl was extracted from the sample leaves (1.1 cm^2) in N,N-dimethylformamide, and then the Chl content in extracts was measured spectrophotometrically (*V630*, *JASCO Corporation*, Tokyo, Japan) according to Porra *et al.* (1989). Other sample leaves (3.4 cm^2) were air-dried for 2 d at 80°C and weighed. LMA was calculated by dividing dry mass by leaf area values. The N content of dried leaf samples (0.3 g) was determined by using a micro-Kjeldahl procedure (Yabiku and Ueno 2017).

Leaf structure and its quantitative analysis: Part of each sample collected from the middle of the leaves was used for structural observation. For the measurement of interveinal distance (IVD), leaf samples (*ca.* 5 × 5 mm) were fixed in a mixture of formalin, acetic acid, and ethanol, and then cleared in saturated chloral hydrate as described in Ueno *et al.* (2006). The IVD in the cleared leaves was measured with a micrometer under an optical microscope (*Eclipse Ci-L*, *Nikon*, Tokyo, Japan) as the distance between the centers of adjacent small vascular bundles. The IVD is represented by the mean of ten measurements per leaf.

Other leaf samples (*ca.* 1 × 1 mm) were fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 1.5 h. After washing with phosphate buffer, they were post-fixed in 2% (w/v) OsO_4 in 50 mM sodium phosphate buffer (pH 6.8) for 2 h. Then they were dehydrated through an acetone series and embedded in *Quetol* resin (*Nisshin-EM Co. Ltd*., Shinjuku, Tokyo, Japan). Transverse sections (*ca.* 1 μm thick) of the leaves were cut with a glass knife on an ultramicrotome (*Reichert Ultracut S*, *Leica*, Wien, Austria), stained with 1% toluidine blue O, and observed under the optical microscope. Leaf thickness and profile area of mesophyll and bundle-sheath (BS) cells were measured on digital

images of the transverse sections by using *ImageJ* software (Schneider *et al.* 2012). Leaf thickness was equated as the mean of ten points per leaf. The areas of mesophyll and BS cells were equated as the means of 20 cells per leaf. The number of chloroplasts per cell was counted for these cells. The area of mesophyll chloroplasts was measured for 24 chloroplasts of six cells per leaf, whereas that of BS chloroplasts was done for 35 chloroplasts of six cells per leaf. The chloroplast occupancy (ratio of chloroplast area to cell area) was calculated from the cell area, the number of chloroplasts per cell, and the chloroplast area (Yabiku and Ueno 2019).

Statistical analysis: We performed a split-plot analysis of variance (*ANOVA*), treating an individual plant as a block, planting density as the main plot, and leaf position as the subplot. Analyses were performed in *PROC GLM v. 9.3* software (*SAS Institute Inc.*, Cary, NC, USA) with the *SAS Studio* interface. The least significant difference (LSD) test was used to determine differences of the means between upper and lower leaves at each planting density.

Results

Gas exchange and photochemical traits of photosynthesis: At SPD, P_N was significantly higher in the upper leaves at PPFD ≥ 500 μmol m^{-2} s^{-1} (Fig. 2*A*). However, P_N tended to be higher in the lower leaves at the low PPFD region (Fig. 2*C*). At LPD, P_N did not differ significantly between upper and lower leaves at any PPFD (Fig. 2*B*,*D*). Similarly, P_N at PPFD = 2,000 μmol m^{-2} s^{-1} (P_{2000}), Φ, LCP, and R_D were significantly higher in the upper leaves than that in the lower leaves at SPD, but did not differ at LPD (Table 1). Φ_{PSII} decreased with increasing PPFD in both upper and lower leaves at SPD and LPD (Fig. 3), although that in the lower leaves at SPD increased slightly from 20 to 60 μmol m^{-2} s^{-1} (Fig. 3*C*). At SPD, Φ_{PSII} was significantly higher in the upper leaves at all PPFDs (Fig. 3*A*,*C*). At LPD, however, it did not differ (Fig. 3*B*,*D*).

Other physiological traits of leaves: At SPD, leaf N content, Chl *a*/*b* ratio, and LMA were higher in upper leaves than that in lower leaves (Table 1). Chl (*a*+*b*) content per leaf area did not differ, but that per dry mass was higher in lower leaves (Table 1). At LPD, leaf N content did not differ between upper and lower leaves, but Chl *a*/*b* ratio and LMA were higher in upper leaves. Chl (*a*+*b*) content per leaf area did not differ between upper and lower leaves, but that per dry mass was higher in lower leaves (Table 1).

Structural traits of leaves: The upper and lower leaves at both SPD and LPD showed almost the same cellular framework, in which an outer layer of mesophyll and an inner layer of BS surrounded the vascular bundle (Fig. 4). The BS cells contained centrifugally located chloroplasts. However, the quantity of chloroplasts in the mesophyll and BS cells was reduced in lower leaves of SPD (Fig. 4*C*). The leaf thickness, IVD, and areas of the mesophyll and BS cells did not differ significantly between upper and lower leaves at SPD or LPD (Table 2).

Quantitative traits of chloroplasts: At SPD, the mesophyll and BS cells had smaller chloroplasts in lower leaves than that in upper leaves, whereas the number of chloroplasts per mesophyll and BS cell did not differ between upper and lower leaves (Table 2). As a result of the decreased area of chloroplasts in lower leaves at SPD, the chloroplast occupancy in the mesophyll and BS cells was reduced in lower leaves as compared to upper leaves (Fig. 4*A*,*C*; Table 2). At LPD, however, there were no differences in the quantitative traits of chloroplasts in the mesophyll and BS cells between upper and lower leaves. (Fig. 4*B*,*D*; Table 2).

Discussion

Leaves in the lower canopy develop under high-light conditions at the early growth stage and then are gradually shaded by upper leaves as the plant grows. To generate lower canopy leaves in different light environments, we grew two maize canopies at different planting densities. At both planting densities, PPFD was lower in the lower leaves than that in the upper leaves (Fig. 1). As expected, DLI in lower leaves was lower at SPD (19% of that in upper leaves) than at LPD (39%). However, the diurnal pattern of PPFD in lower leaves differed greatly between densities: weakly fluctuating PPFD at SPD but greatly fluctuating PPFD with several long sunflecks at LPD.

The light-response curves of photosynthesis of upper leaves at SPD and both upper and lower leaves at LPD

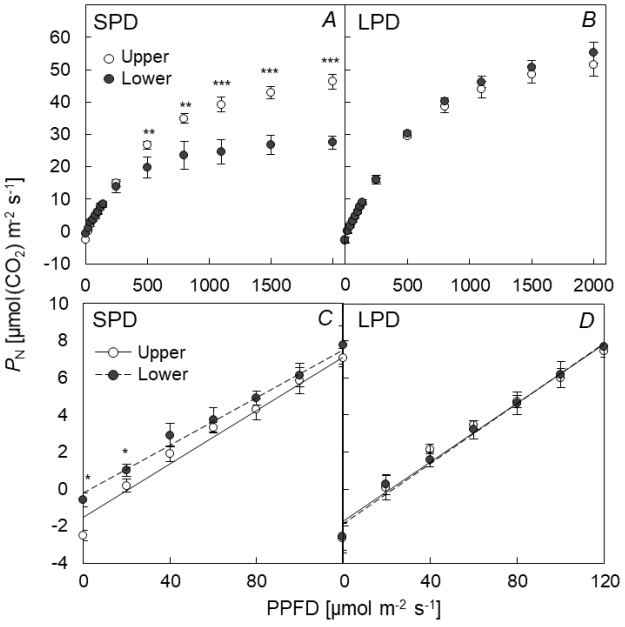

Fig. 2. Response of net photosynthetic rate (P_N) to photosynthetic photon flux density (PPFD) of upper and lower leaves in maize canopies grown at (*A*,*C*) standard (SPD) or (*B*,*D*) low planting density (LPD) under (*A*,*B*) full light or (*C*,*D*) low light. Means ± SD (n = 3 or 4). Significant difference at $^{*}P<0.05$, $^{**}P<0.01$.

Table 1. Comparison of physiological and structural traits of upper and lower leaves of maize at standard (SPD) and low (LPD) planting density. Values are means ± SD (n = 3 or 4). *Asterisks* indicate significant difference between upper and lower leaves at each density by LSD test at $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns – not significant. Symbols for *ANOVA* as in the LSD test except + ($P<0.10$). Chl – chlorophyll; LCP – light-compensation point; LMA – leaf mass per area; LP – leaf position; N – nitrogen; P_{2000} – net photosynthetic rate at PPFD = 2,000 µmol m^{-2} s^{-1}; PD – planting density; R_D – dark respiration rate; Φ – quantum yield of CO_2 fixation.

Traits	SPD			LPD			*ANOVA*		
	Upper leaf	Lower leaf	Low/Up	Upper leaf	Lower leaf	Low/Up	PD	LP	PD × LP
P_{2000} [µmol(CO_2) m^{-2} s^{-1}]	43.5 ± 5.8	26.0 ± 3.2**	0.60	51.6 ± 3.6	55.2 ± 3.2^{ns}	1.03	**	**	***
Φ [mol(CO_2) mol(photon)$^{-1}$]	0.073 ± 0.004	0.060 ± 0.003*	0.82	0.072 ± 0.004	0.074 ± 0.004^{ns}	1.03	+	*	**
LCP [µmol(photon) m^{-2} s^{-1}]	22.1 ± 2.9	7.5 ± 5.1*	0.34	23.2 ± 5.1	23.9 ± 6.5^{ns}	1.03	*	+	*
R_D [µmol(CO_2) m^{-2} s^{-1}]	1.80 ± 0.28	0.45 ± 0.45*	0.25	2.08 ± 0.64	2.04 ± 0.61^{ns}	0.98	*	+	+
Leaf N content [mmol m^{-2}]	78.6 ± 5.7	58.2 ± 10.7**	0.74	101.0 ± 5.1	92.1 ± 9.6^{ns}	0.91	**	**	ns
Chl (a+b) [mg m^{-2}]	507 ± 47	481 ± 72^{ns}	0.95	612 ± 57	722 ± 58^{ns}	1.18	*	ns	+
Chl (a+b) [mg g^{-1}(DM)]	9.0 ± 0.3	12.3 ± 0.4*	1.37	9.5 ± 1.6	15.5 ± 1.5**	1.63	ns	**	ns
Chl a/b	4.65 ± 0.08	3.93 ± 0.26***	0.85	4.92 ± 0.04	4.01 ± 0.16***	0.81	ns	***	ns
LMA [g m^{-2}]	56.0 ± 3.7	38.9 ± 5.0***	0.69	65.7 ± 5.7	46.6 ± 3.3***	0.71	***	***	ns

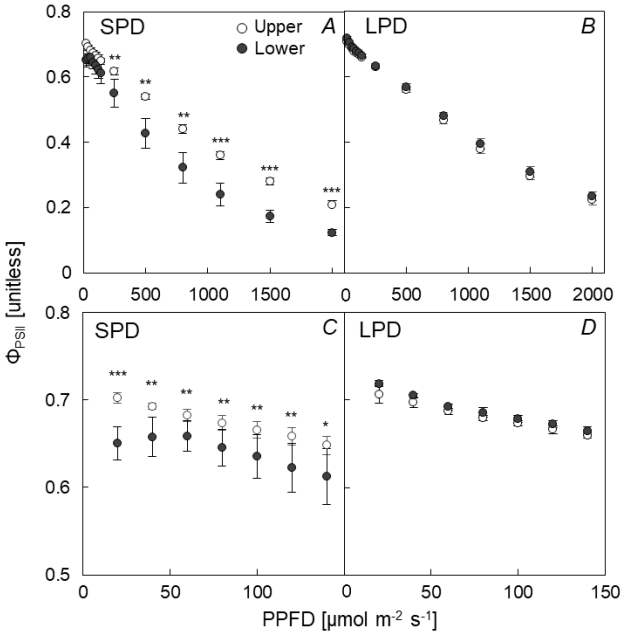

Fig. 3. Response of quantum yield of photosystem II (Φ_{PSII}) to photosynthetic photon flux density (PPFD) of upper and lower leaves in maize canopies grown at (*A*,*C*) standard (SPD) or (*B*,*D*) low planting density (LPD) under (*A*,*B*) full light or (*C*,*D*) low light. Means ± SD (n = 3 or 4). Significant difference at $^{*}P<0.05$, $^{**}P<0.01$.

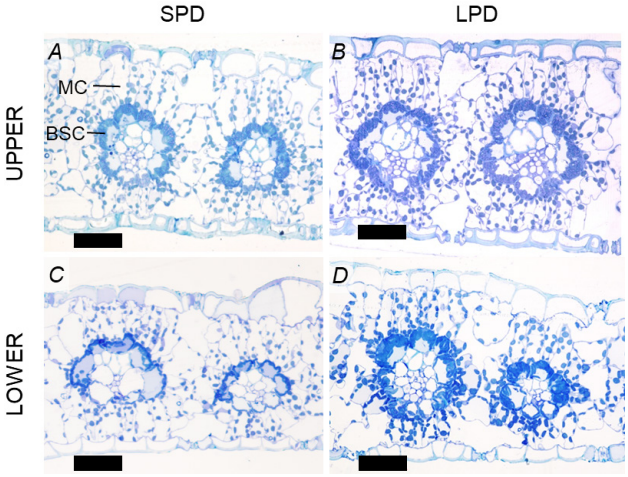

Fig. 4. Transverse sections of (*A*,*B*) upper and (*C*,*D*) lower leaves in maize canopies grown at (*A*,*C*) standard (SPD) or (*B*,*D*) low planting density (LPD). BSC – bundle sheath cell; MC – mesophyll cell. Scale bars = 50 µm.

were typical of sun leaves, whereas that of lower leaves at SPD was typical of shade leaves (Fig. 2). The former had higher values of P_{2000}, LCP, and R_D than the latter (Table 1), as it is typical of sun and shade leaves (Björkman 1981, Givnish 1988, Sage and McKown 2006, Lambers *et al.* 2008). Recently, some studies have reported similar results in pot-grown maize plants; leaves developed under high light and then shaded showed photosynthetic traits of shade leaves, whereas leaves developed and maintained under high light retained those of sun leaves (Bellasio and Griffiths 2014a,b; Yabiku and Ueno 2019). Therefore, our results obtained here show that the reacclimatization of photosynthetic traits to a low-light environment occurs in the lower leaves of a maize canopy as well.

It is interesting to note that lower and upper leaves at LPD had almost the same light-response curve of photosynthesis (Fig. 2*B*,*D*), despite the big reduction in DLI in lower leaves (Fig. 1*B*). Poorter *et al.* (2019) have pointed out the importance of DLI in plant responses to light intensity. On the other hand, Yin and Johnson (2000) and Alter *et al.* (2012) have suggested that sunflecks are involved in acclimatization of photosynthetic traits of plants to light intensity. Our results that the PPFD in the lower leaves fluctuated widely during the day also suggest that this, rather than DLI, is responsible for the photosynthetic traits expressed in the lower leaves at LPD.

Table 2. Comparison of structural and chloroplastic traits of upper and lower leaves of maize at standard (SPD) and low (LPD) planting density. Values are means ± SD (n = 3 or 4). *Asterisks* indicate significant difference between upper and lower leaves at each density by LSD test at $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns – not significant. Symbols for *ANOVA* as in the LSD test except + ($P<0.10$). BSC – bundle sheath cell; IVD – interveinal distance; MC –mesophyll cell.

Traits	Cell	SPD			LPD			*ANOVA*		
		Upper leaf	Lower leaf	Low/Up	Upper leaf	Lower leaf	Low/Up	PD	LP	PD × LP
Leaf thickness [μm]		181 ± 10	176 ± 20^{ns}	0.97	219 ± 7	205 ± 11^{ns}	0.94	*	+	ns
IVD [μm]		131 ± 8	140 ± 6^{ns}	1.07	139 ± 7	141 ± 3^{ns}	1.01	ns	+	ns
Cell area [μm^2]	MC	477 ± 65	519 ± 134^{ns}	1.09	554 ± 49	680 ± 137^{ns}	1.23	ns	ns	ns
	BSC	420 ± 50	441 ± 54^{ns}	1.05	553 ± 37	564 ± 103^{ns}	1.02	**	ns	ns
Chloroplast area [μm^2]	MC	19.7 ± 1.6	15.0 ± 1.0*	0.76	19.0 ± 1.7	19.9 ± 1.2^{ns}	1.05	**	+	*
	BSC	33.4 ± 2.1	21.0 ± 0.5**	0.63	35.9 ± 4.6	35.4 ± 6.4^{ns}	0.99	*	*	*
Chloroplast number [cell^{-1}]	MC	7.4 ± 0.5	7.5 ± 1.6^{ns}	1.01	8.8 ± 1.1	8.3 ± 0.6^{ns}	0.95	ns	ns	ns
	BSC	5.9 ± 0.9	5.6 ± 0.2^{ns}	0.97	5.8 ± 0.4	6.6 ± 0.4^{ns}	1.14	*	ns	*
Chloroplast occupancy [%]	MC	31.3 ± 4.2	22.3 ± 2.2*	0.71	30.7 ± 5.6	25.8 ± 4.1^{ns}	0.84	ns	*	ns
	BSC	56.3 ± 6.2	40.8 ± 2.7**	0.72	57.0 ± 1.5	51.5 ± 6.9^{ns}	0.90	*	*	ns

Φ represents the quantum efficiency of CO_2 uptake at low light and is therefore critical to explaining canopy photosynthesis (Long *et al.* 1993, Ehleringer *et al.* 1997). At SPD, Φ was lower in lower leaves than that in upper leaves (Table 1), as reported also in canopies of maize and *Miscanthus* × *giganteus* (Kromdijk *et al.* 2008, Pignon *et al*. 2017). On the other hand, conflicting reports of the response of Φ in low-light plants *vs*. high-light plants (Ehleringer and Pearcy 1983, Ward and Woolhouse 1986, Beyschlag *et al.* 1990, Lambers *et al.* 2008, Tazoe *et al.* 2008) suggest that the response of Φ to light environment may be affected by species and various factors such as light quality and leaf aging. Φ_{PSII}, which represents the quantum efficiency of PSII, was also lower in lower leaves at SPD (Fig. 3*A*). But in contrast to Φ, there are few studies on the response of Φ_{PSII} during light acclimatization. Brugnoli *et al.* (1998) reported that in maize (C_4) and *Hedera helix* (C_3), shade leaves have lower Φ_{PSII} than the sun leaves. Zheng *et al.* (2011) also showed that Φ_{PSII} decreased with increased shading in the canopy of winter wheat. Therefore, it is suggested that the lower Φ_{PSII} in lower leaves at SPD was caused by mutual shading of leaves. In maize, it seems that the photochemical efficiency of PSII is not enhanced during light reacclimatization in lower leaves at SPD, as in Φ.

In general, shade leaves have a lower leaf N content per unit area and lower LMA than that of sun leaves (Björkman 1981, Lambers *et al.* 2008). These differences between lower and upper leaves were seen also at both SPD and LPD, with the exception of leaf N content at LPD (Table 1). The Chl (a+b) content per dry mass is higher in shade leaves than that in sun leaves, but the content per leaf area is similar between shade and sun leaves (Lambers *et al.* 2008). Similar trends were also found between lower and upper leaves at SPD (Table 1). On the other hand, the Chl a/b ratio is lower in shade leaves than that in sun leaves (Björkman 1981, Lambers *et al.* 2008), as seen at SPD (Table 1). These responses of Chl parameters would make it possible for plants growing under low light to capture light efficiently (Björkman 1981, Givnish 1988, Lambers *et al.* 2008), as in the lower leaves of a crop canopy. This explains the decrease in Chl a/b ratio with decreasing light intensity observed in many crop species (Evans 1993, Li *et al.* 2010). On the other hand, in spite of the shade responses of Chl parameters and LMA (Table 1), the lower leaves at LPD had a light-response curve of photosynthesis typical of sun leaves (Fig. 2*B*). A possible explanation is that different types of light reacclimatization occurred in lower leaves at LPD: the Chl parameters reacclimatized to DLI, whereas P_N reacclimatized to periodic high PPFD. Further studies would be required to reveal whether the light environment in the lower leaves at LPD is involved in this phenomenon.

Shade leaves are thinner than sun leaves; this allows shade leaves to capture weak light efficiently in shade (Björkman 1981, Lambers *et al.* 2008). However, the lower leaves in a canopy are likely to have unique anatomical traits, because they develop under high light and are then progressively shaded. Therefore, they start with the biochemical and physiological traits of sun leaves, which change gradually to those of shade leaves, still within the anatomical structure of sun leaves, as found in our study of pot-grown maize plants (Yabiku and Ueno 2019), as well as in lower leaves of field-grown maize canopies. The lower leaves at both SPD and LPD had almost the same anatomical framework as the upper leaves had (Fig. 4), with similar leaf thickness, IVD, and cell area (Table 1). However, the area and occupancy of chloroplasts in the mesophyll and BS cells were reduced in the lower leaves at SPD (Fig. 4*C*, Table 2). As expected of photosynthetic traits typical of sun leaves, the lower leaves at LPD maintained abundant chloroplasts in the mesophyll and BS cells (Fig. 4*D*). These results corresponded to those found in our shading experiment of pot-grown maize plants (Yabiku and Ueno 2019).

In this study, we did not consider the effect of light

quality on the light reacclimatization in lower leaves of maize canopies. The lower leaves of canopy in the field are also affected by the change of light quality that was generated by light absorption of upper leaves in a canopy (Pons 2016). Therefore, further studies will be required giving careful consideration to the effects of light quality as well as light intensity.

Conclusion: We investigated the reacclimatization of lower leaves in maize canopies that formed under high light and then became shaded with canopy growth. These lower leaves had the anatomical framework of sun leaves, but the chloroplast contents in mesophyll and BS cells were greatly reduced. Photosynthetic and other physiological traits adjusted to low light. These results are consistent with those of our recent study of pot-grown maize (Yabiku and Ueno 2019). Our study provides useful knowledge for understanding of the performance of C_4 photosynthesis in maize canopies.

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