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Dorsoventral variation in photosynthesis during leaf senescence probed by chlorophyll *a* fluorescence induction kinetics in cucumber and maize plants

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Abstract

Although photosynthesis has been intensively studied during leaf senescence, it is unclear whether the changes of photosynthetic function in various mesophyll tissues can be detected using chlorophyll *a* fluorescence induction kinetics (CFI) *in vivo*. To clarify this question, leaf structure and CFI were carefully examined in senescent leaves. The results showed declines in chlorophyll content and photosynthetic rate in both cucumber and maize plants as leaves aged. In cucumber leaves, the number of chloroplasts decreased more rapidly in the palisade tissue compared to the sponge tissue as senescence progressed. Under various pulse light intensities, the enhancement of the relative variable fluorescence in the CFI curves measured on the adaxial side was larger than that on the abaxial side of senescent cucumber leaves, indicating senescence in the palisade tissue progressed more quickly than that in the sponge tissue. JIP-test parameters further showed that the loss of photosynthetic electron transport activity in palisade tissue was faster than that in sponge tissue. However, during leaf senescence in maize, there were no such dorsoventral differences. We therefore propose that the decline of photosynthesis in cucumber leaves is largely determined by the senescence of palisade tissue. The changes in photosynthesis in palisade and sponge tissues with leaf aging can be probed effectively by CFI measured on both sides of cucumber leaves *in vivo*.

Additional key words: dorsiventral leaf; isobilateral leaf; light gradient; microstructure; photosystem II.

Introduction

During leaf senescence, a gradual decrease of photosynthetic rate has been observed. The number of chloroplasts decreases, the grana lamellae and matrix lamellae begin to disintegrate, and finally the chloroplasts are completely degraded (Krupinska and Humbeck 2004, Martínez *et al*. 2008, Uzelac *et al*. 2016). Chlorophyll (Chl) content therefore declines step by step (Brouwer *et al.* 2014, Oda-Yamamizo *et al*. 2016, Tamary *et al.* 2019). It has been shown that the electron transfer rate reduces during the initial phase of senescence, mainly due to a decreased content of D1 protein and cytochrome (Cyt) b_6f (Holloway *et al*. 1983, Genty *et al*. 1989, Nath *et al*. 2013). Leaf senescence is also accompanied by decreasing activity and content of enzymes in the Calvin cycle, with Rubisco declining most rapidly (Ishizuka *et al*. 2004, Tholen *et al*. 2007, Bi *et al*. 2017, Krieger-Liszkay *et al*. 2019).

Although the regulation of leaf senescence has been intensively studied, little attention has been paid to the different actions occurring in various mesophyll tissues or cells. The photosynthetic function in dorsiventral leaves is mainly determined by palisade tissue cells, while the photosynthetic function of isobilateral leaves may depend on mesophyll tissues of both adaxial and abaxial sides (Soares *et al*. 2008, Soares-Cordeiro *et al*. 2009). Although it has been established that there are large differences in photosynthetic function among various mesophyll tissues of leaves, most previous studies were performed *in vitro* (Terashima and Inoue 1985, Vogelmann and Han 2000, Evans and Vogelmann 2006, Pantaleoni *et al.* 2009, Borsuk and Brodersen 2019). Some investigators have examined the difference in photosynthetic function between mesophyll tissues on adaxial and abaxial sides of isobilateral leaves *in vivo* by improving the gas-exchange system, but this method cannot eliminate the diffusion of

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Abbreviations: Chl – chlorophyll; C_i – intercellular CO_2 concentration; E – transpiration rate; g_s – stomatal conductance; P_N – net photosynthetic rate; RC/CS_0 – the density of PSII reaction centers; S_m – the normalized area over the OJIP transient curve; S_{mc} – area of chloroplast surface in mesophyll cells per leaf area (cm^2); φ_{E0} – the quantum yield of electron transport beyond Q_A; φ_{P0} – the maximum quantum yield of primary photochemistry.
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CO_2 vertically within leaves (Driscoll *et al.* 2006, Soares *et al.* 2008, Soares-Cordeiro *et al*. 2009). Hence, improved gas exchange could not reveal the real photosynthetic differences in the dorsoventral mesophyll tissues. Moreover, dorsiventral leaves have more pronounced CO_2 diffusion vertically within leaves. These limitations make this method unsuitable for studying the photosynthetic function of dorsoventral mesophyll tissue in dorsiventral leaves. Thus, there is a need for an effective and rapid method to detect differences in photosynthetic function between adaxial and abaxial sides *in vivo*.

Chl *a* fluorescence induction kinetics (CFI) is a rapid and noninvasive detection method that has been widely used to analyze photosynthetic function. Red light is mostly used as an excitation light in CFI. Because the absorption of red light in plant leaves is very high, it is generally believed that red light is almost completely absorbed by Chl in the shallow cells of leaves and rarely reaches the deep cells. Therefore, our aim was to clarify whether the changes of photosynthetic function in various mesophyll tissues with leaf senescence can be detected using CFI *in vivo*, thus revealing the dorsoventral variations of various mesophyll tissues during leaf senescence.

To solve these questions, we studied changes in chlorophyll content, photosynthetic rate, leaf structure, and chlorophyll *a* fluorescence induction kinetics during leaf senescence in maize and cucumber seedlings.

Materials and methods

Plant materials and experimental design: Experiments were carried out in 2018 at the Institute of Botany at the Chinese Academy of Sciences in Beijing (39°28'–41°25'N, 115°25'–117°30'E). Meteorological data for 2018 in Beijing were obtained from meteorological stations located near the experimental sites; the mean annual temperature was 13.5°C, the mean annual light duration was 7 h, the total annual precipitation was 575.5 mm. The isobilateral leaf maize (*Zea mays* L., Zhengdan958) and dorsiventral leaf cucumber (*Cucumis sativus* L., Jinyou35) were used in this study. Zhengdan958 is a hybrid of Zheng58 as the female parent and Chang7-2 as the male parent. Jinyou35 is a hybrid of X8202 as the female parent and G3515 as the male parent. Maize and cucumber seeds were imbibed on wet culture dishes for 72 h and the germinated seeds were sown in pots (20 cm in height and 17 cm in diameter) with seepage pores filled with a 1:1 mixture of loess and peat. Potted seedlings were cultured in the field. Normal water and fertilizer management was carried out throughout to avoid potential nutrient and drought stresses. The third leaf (from top to bottom) was used for all measurements in this study. When the third leaves of maize and cucumber were fully expanded, the Chl content, Chl *a* fluorescence induction kinetics, and gas exchange were measured, and semi-thin slices of the leaves were made at 0, 7, 14, 21, and 28 d after the leaves had fully expanded.

Chl content: Pigments extracted in 80% acetone were determined using a previously described method (Arnon 1949) with small modifications. Each treatment was repeated at least six times using six plants. Control tubes contained 10 mL of 80% acetone. Test tubes were placed in the dark for 48 h, oscillated every 12 h, and then mixed evenly after the green of the leaf disc completely faded. The absorbance at 663, 645, and 440 nm was determined by ultraviolet-visible spectrophotometer (*UV-8000S*, China) and the pigment content was calculated.

Leaf gas-exchange parameters: Gas exchange was measured with a CO_2 concentration of 360–400 μmol mol^{-1} at 70–80% relative humidity under an irradiance of 2,000 μmol(photon) m^{-2} s^{-1} using a portable photosynthesis system (*Ciras-2*, *PP Systems*, USA). The net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), and intracellular CO_2 concentration (C_i) were determined before 12:00 h on a sunny day. Six replicates were measured for each period.

Chl *a* fluorescence induction kinetics: As described by Jiang *et al*. (2006), Chl *a* fluorescence induction kinetics (CFI) was measured with a *Handy Plant Efficiency Analyzer* (*Hansatech*, UK). Fully dark-adapted leaves (> 30 min) were used to determine the Chl *a* fluorescence transient of adaxial and abaxial sides at 20:00 h. The CFI in adaxial and abaxial sides was recorded during 1-s pulses of red radiation at a range of intensities [200–1,000 and 2,000–3,000 μmol(photon) m^{-2} s^{-1}] provided by an array of six light-emitting diodes. Twenty leaves were measured for each treatment.

All of the oxygenic photosynthetic materials exhibit a polyphasic fluorescence rise from initial fluorescence intensity (F_0) to maximum fluorescence intensity (F_m) upon illumination. These phases are labeled as O, J, I, and P (Strasser *et al.* 2000, 2004; Force *et al.* 2003). Recently, a quantitative analysis of O-J-I-P transient has been developed by Strasser *et al*. (2000, 2004) and called the JIP-test after the basic steps of the transient. The JIP-test has proven to be a powerful tool for the *in vivo* investigation of the structure and function of PSII. In this study, the following original data were obtained: maximum chlorophyll fluorescence yield (F_p), which was equal to F_m since the excitation intensity was high enough to ensure the closure of all reaction centers of PSII; the fluorescence intensity at 50 μs, which was considered as the minimum intensity (F_0) when all reaction centers are open; the fluorescence intensity at 2 ms and 30 ms, denoted as F_J and F_I, respectively. To further reveal the changes of PSII function in cucumber leaves, JIP-test parameters were calculated according to Strasser *et al.* (2000, 2004). The normalized area over the OJIP transient curve (S_m), the maximum quantum yield of primary photochemistry (φ_{P0}), the quantum yield of electron transport beyond Q_A (φ_{E0}), and the density of PSII reaction centers (RC/CS_0)were calculated according to Strasser *et al.* (2000, 2004): $\varphi_{E0} = ET_0/ABS = [1 - (F_0/F_m)]\Psi_0$, $\varphi_{P0} = TR_0/ABS = [1 - (F_0/F_m)]$, $S_m = (Area)/(F_m - F_0)$, $RC/CS_0 = \varphi_{P0}(V_J/M_0)(ABS/CS_0)$.

Leaf structure and chloroplast distribution: Semi-thin leaf sections were made and observed with reference

to a method described by Gong *et al*. (2014) and Jiang *et al*. (2011). Leaf segments (2 × 3 mm) without major veins were cut from the intermediate part of the leaf with a razor blade at 5:00 h. The segments were fixed at 4°C in 3% glutaraldehyde which was formulated with 8% glutaraldehyde, 0.1 mol L^{-1} phosphate buffer, and distilled water. Each segment was pumped by vacuum to completely immerse it. During sample preparation, the segment was rinsed with phosphate buffer solution three times, then fixed in 1% osmic acid solution for 6 h, then again rinsed three times with 0.1 mol L^{-1} phosphate buffer, then dehydrated in a graded series of ethanol and acetone solutions and embedded in *Spurr*'s resin (*Ladd Research Industries*, USA). Light microscopy was carried out with 1-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. Leaf structure and chloroplast distribution were observed and photographed with a light microscope (*Nikon-E800*, *Scientific Imaging Inc*, USA).

The adaxial and abaxial chloroplast area was obtained using *Photoshop* software. Twenty sections were measured.

Statistical analysis: Data were analyzed using one-way analysis of variance (*ANOVA*) and compared with the significant difference (LSD) multiple comparison test using *SPSS* (*version 25*). The significant differences refer to statistical significance at $P<0.05$. The graphics software *SigmaPlo*t (*version 12.5*) was used to create artwork.

Results

Chl content: The Chl content of maize and cucumber leaves decreased gradually during leaf senescence (Fig. 1*A*). At the early stage of senescence, the Chl content declined slightly, and then the decrease accelerated at the later stage of senescence. A similar pattern was observed with carotenoid. Although both Chl and carotenoid contents reduced significantly, Chl demonstrated the greater decrease than that of carotenoids.

Gas-exchange parameters: As maize and cucumber leaves became senescent, we observed a clear reduction in P_N (Fig. 2*A*), g_s (Fig. 2*B*), and E (Fig. 2*C*), whereas intracellular CO_2 concentration (C_i) gradually increased (Fig. 2*D*), indicating that the increase in C_i was due to a decrease in CO_2 assimilation. During the first 7 d, the P_N, g_s, and E dropped only slowly, but after that the decrease became rapid.

Microstructure: Changes in the light micrographs of cross sections of maize and cucumber leaves are shown in Fig. 3. The surface area of chloroplasts in mesophyll cells per leaf cross section (S_{mc}) represents the number of chloroplasts (Gong *et al.* 2014). In this study, S_{mc} of maize and cucumber leaves decreased with aging. The values of S_{mc} in mesophyll tissues of leaf adaxial and abaxial sides declined markedly by the 14th d after full expansion of maize leaves and almost vanished by 28 d (Fig. 4). Moreover, in maize leaves, there was almost no difference between adaxial and abaxial sides. However, by the 14th d after full expansion, the S_{mc} in the palisade tissue (leaf adaxial side) and sponge tissue (leaf abaxial side) of cucumber leaves decreased by 42.0 and 33.0%, respectively. Obviously, the decrease of S_{mc} in the palisade tissue was significantly higher than that in sponge tissue (Fig. 4). These results indicated that there were pronounced differences in leaf senescence between maize and cucumber seedlings.

Chl *a* fluorescence induction kinetics: Fig. 5 and Fig. 6 show the changes in CFI curves in maize and cucumber plants. With leaf aging, the relative fluorescence yields of the J and I phases in adaxial and abaxial sides of maize leaves increased significantly when measured by either weak or high pulse light intensity, especially the J phase (Fig. 5). In cucumber leaves, the relative fluorescence yields of the J and I phases in the adaxial side increased significantly under the weak or high pulse light intensity, while this was not observed on the abaxial side (Fig. 6). These results suggest that there may be pronounced dorsoventral differences in senescence between maize and cucumber leaves.

In order to compare the CFI of leaf adaxial and abaxial sides at different stages of senescence, the data was reanalyzed. As shown in Fig. 7, we observed little difference in CFI between the adaxial and abaxial sides of maize leaves, regardless of whether they were measured

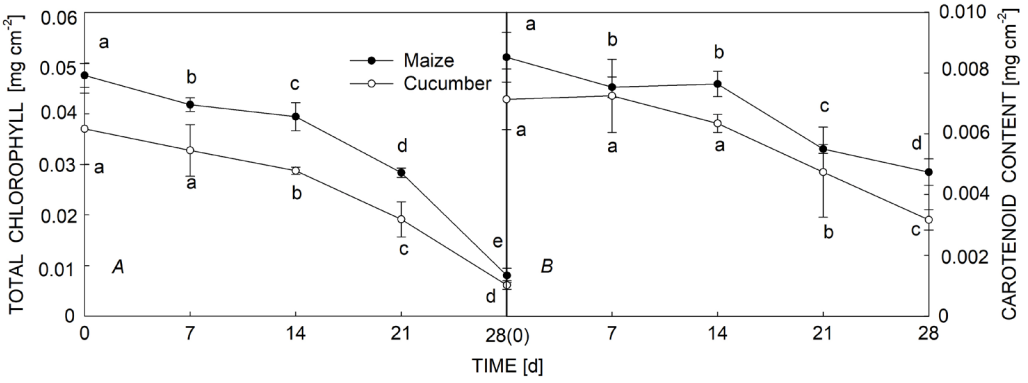

Fig. 1. Changes of total chlorophyll (*A*) and carotenoid (*B*) contents during leaf senescence in maize and cucumber plants. Data are means ± SE ($n = 5$), *different lowercase letters* indicate statistically significant differences at the $P<0.05$ level.

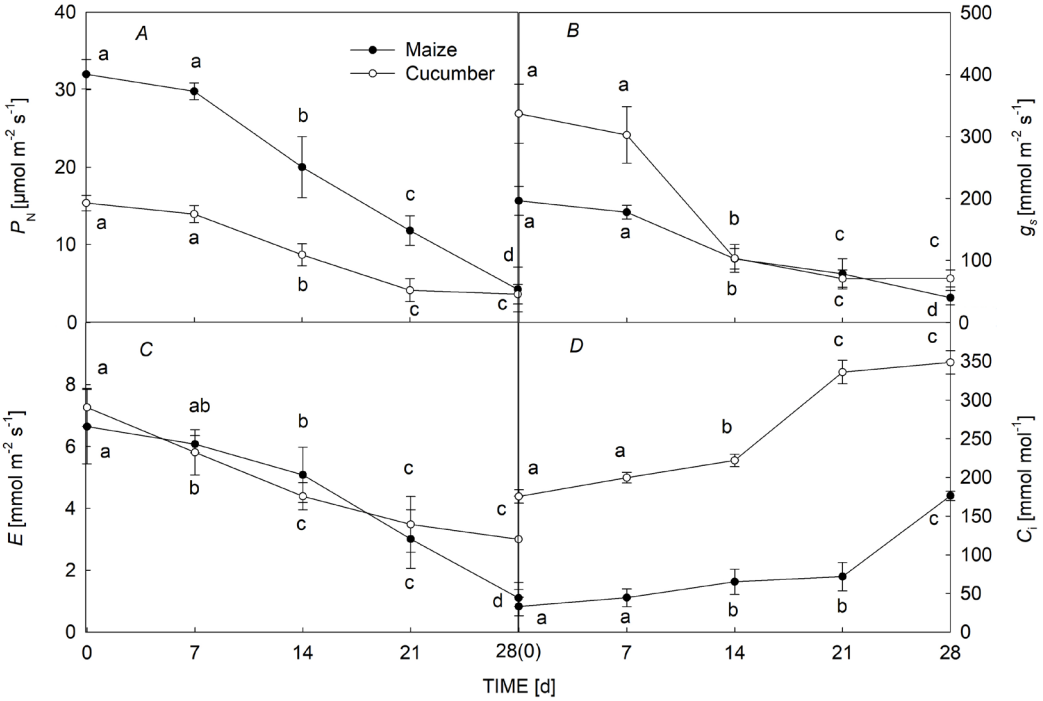

Fig. 2. Changes of net photosynthetic rate (P_N) (*A*), stomatal conductance (g_s) (*B*), transpiration rate (*E*) (*C*), and intercellular CO_2 concentration (C_i) (*D*) during leaf senescence in maize and cucumber plants. Data are means ± SE (n = 6), *different lowercase letters* indicate statistically significant differences at the P<0.05 level.

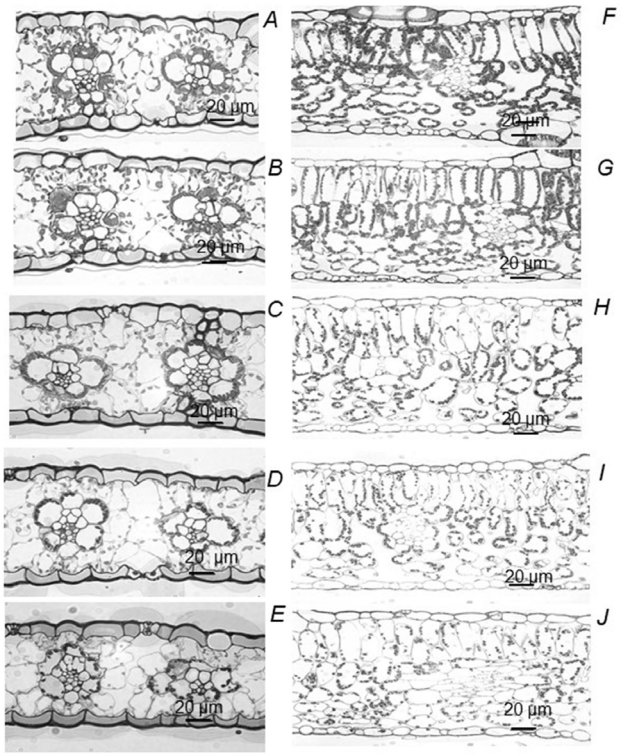

Fig. 3. Changes of microstructures during leaf senescence in maize (*left*) and cucumber (*right*) plants. On the day of full leaf expansion (*A*,*F*); on the 7th (*B*,*G*), 14th (*C*,*H*), 21st (*D*,*I*), and 28th (*E*,*J*) day after full leaf expansion.

by weak or high pulse light intensity (Fig. 7*A–C*). However, in cucumber leaves, the relative fluorescence yields of the J and I phases measured on the adaxial side were considerably lower than those on the abaxial side at the beginning of senescence, as measured by various pulse light intensities. With the decrease of pulse light intensity, the difference still existed. Therefore, there was an apparent difference in CFI between the adaxial and abaxial sides of cucumber leaves. As cucumber leaf senescence progressed, the difference in the relative fluorescence yields of the J and I between the leaves adaxial and abaxial sides lessened (Fig. 7*D–F*).

As shown in Fig. 8, the maximum quantum yield of primary photochemistry (φ_{P0}) and the quantum yield of electron transport beyond Q_A (φ_{E0}) of leaf adaxial and abaxial sides in cucumber leaves decreased slightly with leaf aging, reflecting that senescent leaves possessed relative high primary photochemistry and electron transport activity of PSII. In addition, senescence also induced the clear decline in the normalized area over the OJIP transient curve (S_m) and the density of PSII reaction centers (RC/CS_0) in both sides of leaves. These data show that the pool of electron acceptors and reaction centers of PSII decreased significantly during leaf senescence.

Discussion

The differences in photosynthetic function between leaf adaxial and abaxial sides: It is well established that leaves have a strong absorption in red region due to high concentration of Chl. When leaves were irradiated with

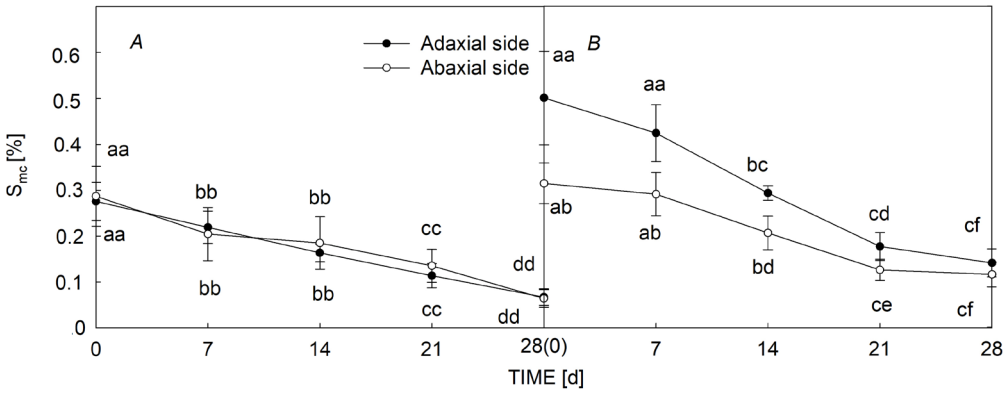

Fig. 4. Changes of the surface area of chloroplast in mesophyll cells per leaf area (S_{mc}) during leaf senescence in maize (*A*) and cucumber (*B*) plants. Data are means ± SE (n = 20), *different lowercase letters* indicate statistically significant differences at the P<0.05 level.

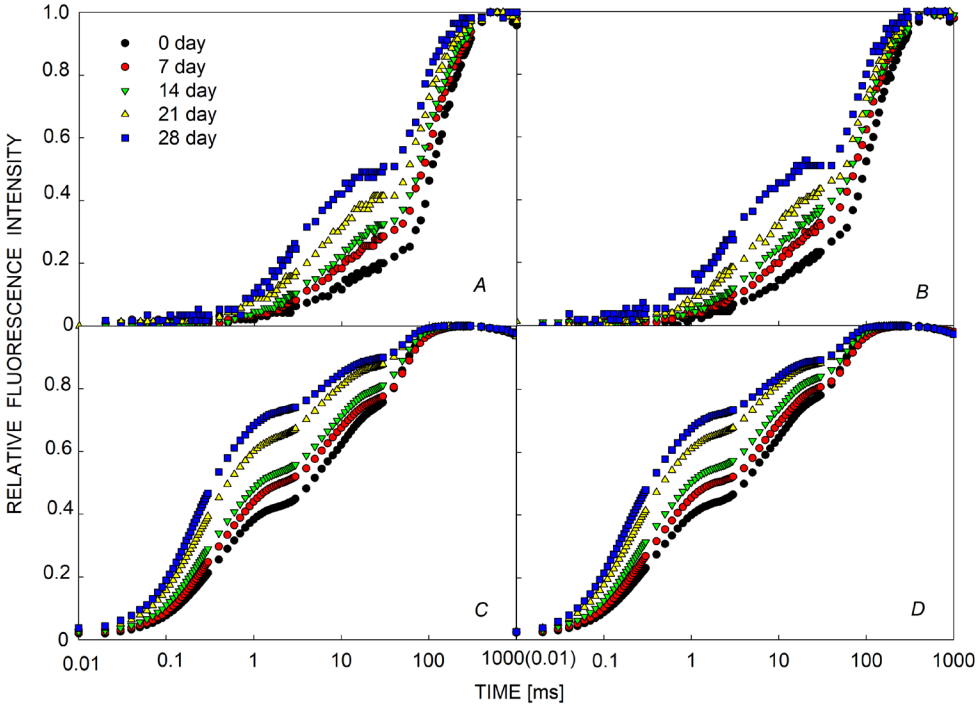

Fig. 5. Changes in chlorophyll *a* fluorescence induction kinetics (CFI) of leaf adaxial and abaxial sides during leaf senescence in maize plants. Day 0 – on the day of full leaf expansion; Day 7, 14, 21, 28 – on the 7th, 14th, 21st, and 28th day after full leaf expansion, respectively. The pulse light intensities were 200 (*A*,*B*) and 3,000 (*C*,*D*) μmol(photon) m^{-2} s^{-1}. The CFI were measured at adaxial (*A*,*C*) and abaxial (*B*,*D*) sides, respectively.

red light, carbon fixation was largely restricted to cell layers near the upper leaf surface, but when leaves were irradiated with green light, the distribution of fixed carbon shifted from the upper surface to the interior of leaf (Sun *et al.* 1998). Therefore, red light penetrates only poorly into leaves (Evans 1999, Vogelmann and Han 2000). Yet, it has been argued that Chl *a* fluorescence induced by red pulse light may originate from deep inside mesophyll cells when a prominent light gradient is present along the path of high pulse light intensity (Hsu and Leu 2003, Sušila *et al.* 2004). Our data on CFI possibly contain an admixture of the signal from the deep inside mesophyll cells or the opposite leaf side. However, as the pulse light intensity decreased, the light gradient within the leaf may have declined, while the difference in CFI curves between the adaxial and abaxial sides of cucumber leaves was still obvious (Fig. 7*D–F*). Even under the weakest pulse light (200 μmol m^{-2} s^{-1}), there was a significant difference in CFI curves between the adaxial and abaxial sides (Fig. 7*D–F*). This demonstrates that the main difference in CFI curves between the adaxial and abaxial sides of cucumber leaves is independent of the light gradient within the leaf under various pulse light intensities. Therefore, we propose that Chl *a* fluorescence originating from deep inside mesophyll cells or the opposite leaf side may not be significant in the present study. That is to say, Chl *a* fluorescence induced

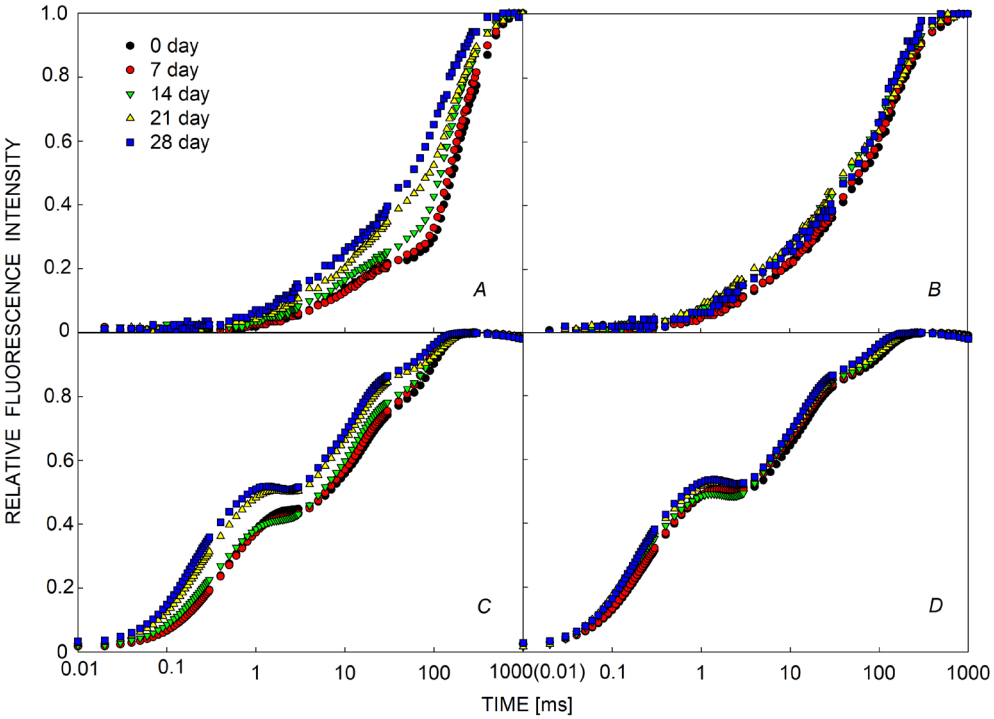

Fig. 6. Changes in chlorophyll *a* fluorescence induction kinetics (CFI) of leaf adaxial and abaxial sides during leaf senescence in cucumber plants. Day 0 – on the day of full leaf expansion; Day 7, 14, 21, 28 – on the 7th, 14th, 21st, and 28th day after full leaf expansion, respectively. The pulse light intensities were 200 (*A*,*B*) and 3,000 (*C*,*D*) μmol(photon) m^{-2} s^{-1}. The CFI were measured at adaxial (*A*,*C*) and abaxial (*B*,*D*) sides, respectively.

by red pulse light is probably mainly generated by the excitation of Chl in shallow mesophyll cells *in vivo*.

In cucumber leaves, there was significant variation in the dorsoventral microstructure. The higher S_{mc} value in palisade tissue than that in sponge tissue during the period before severe senescence (Fig. 3) implies that the former had higher photosynthetic activities. At this time, the relative fluorescence yields of the J and I phases in CFI were significantly lower in the adaxial side than that in the abaxial side (Fig. 7*D–F*). The parameters derived from CFI show that mesophyll tissue at the adaxial side of the leaf had higher φ_{P0}, φ_{E0}, S_m, and RC/CS_0 than that of the abaxial side, demonstrating that the chloroplasts of palisade tissue exhibited higher photosynthetic activity. Our *in vivo* results are consistent with those observed *in vitro* previously (Terashima and Inoue 1985, Vogelmann and Han 2000, Evans and Vogelmann 2006, Pantaleoni *et al.* 2009, Borsuk and Brodersen 2019). In contrast, in isobilateral maize leaves, little difference in dorsoventral microstructure was observed (Figs. 3, 4). Moreover, there was little difference in the CFI curves between the adaxial and abaxial sides of maize leaves, regardless of whether the measurement was made with weak or high pulse light intensity (Fig. 7*A–C*). These results are similar to the fluorescence measurement of other plants, including rice and cotton (unpublished data). Therefore, the difference in photosynthetic activity of palisade and sponge tissues in cucumber leaves may be assessed using CFI *in vivo*.

Variations in photosynthetic function between leaf adaxial and abaxial sides during leaf senescence: In our study, the photosynthetic rate and stomatal conductance decreased in senescent leaves, while the intracellular CO_2 concentration increased (Fig. 2), indicating that photosynthetic activity declined in both cucumber and maize leaves during leaf senescence. Nevertheless, as leaf senescence progressed, the changes in photosynthetic activity in various mesophyll tissues or cells are still unclear *in vivo*.

Previous studies have reported that senescence induces decline in PQ size and photosynthetic electron transport in leaves (Holloway *et al*. 1983, Prakash *et al.* 2003, Nath *et al*. 2013). However, in the present study, the decrease in the content of Chl and the number of chloroplasts in cucumber leaves mainly occurred on the adaxial side (Figs. 1, 3, 4); the decrease in φ_{E0} on adaxial side was more obvious than that on abaxial side as senescence progressed (Fig. 8), demonstrating that palisade tissue in cucumber leaves lost more photosynthetic electron transport activity. We therefore propose that the decrease of photosynthetic capacity in cucumber leaves depends primarily on the senescence of palisade tissue in the adaxial side during leaf senescence. Moreover, with leaf aging, both S_m and RC/CS_0 in the adaxial side of cucumber leaves significantly decreased. Consequently, it appears that the loss of photosynthetic electron transport activity in palisade tissue during leaf senescence mainly results from the decreased pool of electron acceptors and PSII reaction centers. For

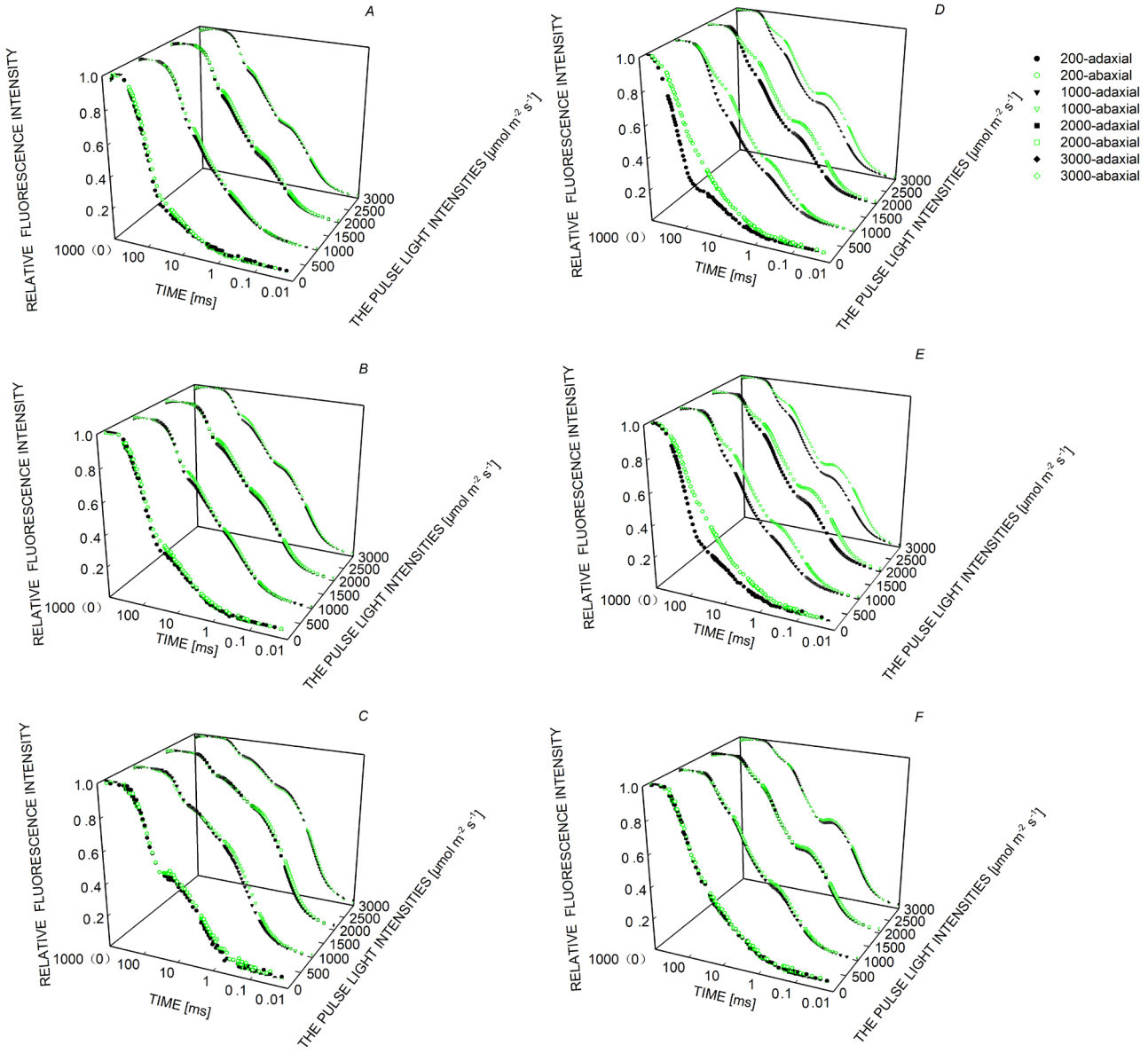

Fig. 7. Differences in chlorophyll *a* fluorescence induction kinetics (CFI) between leaf adaxial and abaxial sides under various light intensities during leaf senescence in maize (*left*) and cucumber plants (*right*). (*A*) and (*D*) represent the initial day of full leaf expansion of maize and cucumber, respectively. (*B*) and (*E*) represent the 14th day after full leaf expansion of maize and cucumber, respectively. (*C*) and (*F*) represent the 28th day after full leaf expansion of maize and cucumber, respectively.

the abaxial side of cucumber leaves, the values of RC/CS_0 decreased more significantly than that of S_m during leaf senescence, so the decline in RC/CS_0 may be responsible for the lowered φ_{E0} in the sponge tissue. However, the φ_{P0} ratio in both the adaxial and abaxial sides was the least sensitive to senescence (Fig. 8). It is likely that the concurrent decreases in light absorption of Chl and trapping of PSII reaction centers per excited cross section resulted in the slight decline in φ_{P0} during leaf senescence.

In maize leaves, the chloroplast decrease was simultaneous at adaxial and abaxial sides, and the difference between the both sides was very small (Figs. 3, 4). Though leaf senescence induced considerable enhancement of the relative fluorescence yields of the J and I phases in the Chl *a* fluorescence induction kinetics curves under various pulse light intensities, there was no significant difference between leaf adaxial and abaxial sides (Figs. 5, 7). This indicated that the decrease of photosynthetic capacity in maize leaves is related to mesophyll tissue in both adaxial and abaxial sides.

Accordingly, we conclude that Chl *a* fluorescence induction kinetic curves reflect the changes in photosynthetic activity on adaxial and abaxial sides of cucumber leaves, and that it is feasible to use the JIP-test to examine the dorsal-ventral photosynthetic differences during leaf senescence *in vivo*.

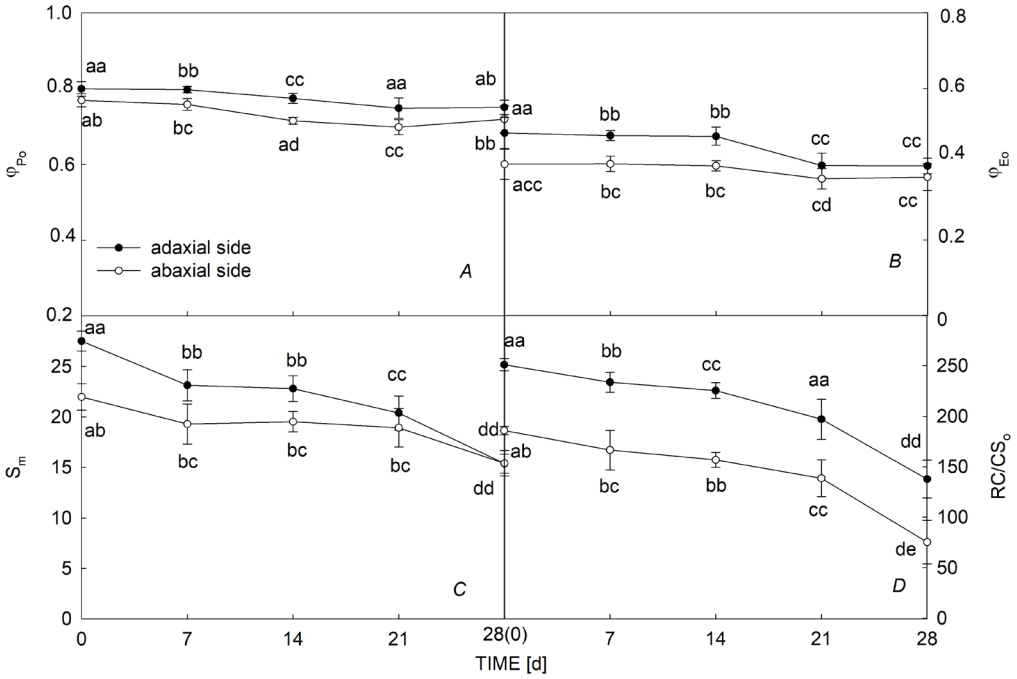

Fig. 8. Changes of the maximum quantum yield of primary photochemistry (φ_{P0}), the quantum yield of electron transport beyond Q_A (φ_{E0}), the normalized area over the OJIP transient curve (S_m), and the density of PSII reaction centers (RC/CS_0) during leaf senescence in cucumber plants. The pulse light intensity was 3,000 μmol(photon) m^{-2} s^{-1}. Data are means ± SE (n = 20), *different lowercase letters* indicate statistically significant differences at the P<0.05 level.

Conclusions: In cucumber plants during the early phase of leaf senescence, there are marked differences between the leaf adaxial and abaxial sides that are revealed not only by changes in leaf microstructures but also by chlorophyll *a* fluorescence induction kinetics. The changes in photosynthetic activity between palisade and sponge tissues in cucumber leaves with leaf aging can be effectively probed by using JIP-test to measure both sides of cucumber leaf *in vivo*.

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