C₃-Like Photosynthetic Properties of Senescing Maize Leaves Are Accompanied by Preferential Senescence of Mesophyll Cells

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Summary In C₄ plants, mesophyll cells (MCs) are engaged in CO₂ pumping into bundle sheath cells (BSCs) to allow ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) to work under CO₂-rich conditions, leading to various properties specific to C₄ photosynthesis. Maize (*Zea mays*) is a representative C₄ plant, but its senescing leaves exhibit C₃-like photosynthetic properties. Under our culture conditions, first leaves of maize finished maturation by day 9 after sowing and then began to exhibit a considerable degree of O₂ inhibition, a C₃-like photosynthetic property. During senescence, the number and size of chloroplast nucleoids decreased in both MCs and BSCs, but the degradation of chloroplast-nucleoids, reportedly one of the earliest signs of senescence in photosynthetic organs, started earlier and proceeded faster in MCs than in BSCs, suggesting preferential senescence of MCs. Based on these results, we propose that preferential senescence in MCs may cause insufficient CO₂ pumping into BSCs and lead to C₃-like photosynthetic properties in senescing maize leaves.

Key words Bundle sheath cell, Mesophyll cell, Chloroplast nucleoid, O₂ inhibition, Photosynthesis, Senescence, Maize.

In C₄ photosynthesis, initial CO₂ fixation by phosphoenolpyruvate carboxylase (PEPC) occurs in MCs. The products (C₄ compounds) are transported into BSCs, where CO₂ is released by their decarboxylation and accumulates to high levels. This situation allows Rubisco in the BSC chloroplasts to work under CO₂-rich conditions, causing various C₄-specific properties, such as high maximum photosynthetic rate, low CO₂ compensation point, and absence of O₂ inhibition (*i.e.*, inhibition of photosynthetic CO₂ fixation under O₂-rich conditions). Maize is a representative NADP-ME type C₄ plant, in which malate transported into BSCs is decarboxylated by the NADP-malic enzyme (NADP-ME); however, older leaves of maize reportedly exhibit an elevated CO₂ compensation point, like a C₃ plant (Crespo *et al.* 1979).

In C₄ plants, BSC and MC chloroplasts play quite different roles; while BSC chloroplasts have Rubisco and perform CO₂ fixation *via* the Calvin cycle, MC chloroplasts do not have Rubisco and are devoted to regeneration of phosphoenolpyruvate (PEP, a CO₂ acceptor in the initial carbon fixation by PEPC) from C₃ compounds returned from BSCs. Furthermore, in NADP-ME type

C₄ plants including maize, BSC and MC chloroplasts are morphologically different; BSC chloroplasts have starch granules but lack grana, whereas MC chloroplasts have well-developed grana but lack starch granules.

Such a striking difference between MC and BSC chloroplasts suggests that the status of chloroplast genomes and chloroplast nucleoids are also different. Chloroplast nucleoids, or, collectively plastid nucleoids, are DNA–protein complexes in chloroplasts and other types of plastids; their structure, localization, and function change drastically during plastid differentiation, *e.g.*, during light-induced differentiation of proplastids to chloroplasts *via* etioplasts (Miyamura *et al.* 1990). Moreover, nucleoids isolated from various plastid types exhibit different protein composition, different DNA content, and different replication/transcription activities (Sakai *et al.* 2004). These facts suggest that plastid differentiation is generally accompanied by changes in the structure and function of plastid nucleoids; therefore, differentiation into MC and BSC chloroplasts should also be. In addition, Sodmergen *et al.* (1989, 1991) and Inada *et al.* (1999, 2002) reported that degradation of chloroplast nucleoids occurs very early during senescence of photosynthetic organs in rice, suggesting their potential usefulness as an initial marker of senescence.

In the present study, therefore, we followed changes in the photosynthetic properties and behavior of chloro-

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plast nucleoids in MCs and BSCs throughout the course of maturation and senescence of first leaves of maize. Based on the results, we propose a possible mechanism for the emergence of C₃-like photosynthetic properties in senescing maize leaves.

Materials and methods

Biological material

Maize (*Z. mays* cv. Canberra 90EX, TAKII & Co., Ltd., Kyoto, Japan) seeds were imbibed overnight and sown on fine vermiculite that was packed in 12-well Jiffy strips (Sakata Seed Corporation, Kanagawa, Japan) in a plastic tray (2 strips per tray) and moistened with 1 L of water, at 2 seeds per well. During culture at 25°C and a 14h light:10h dark photoperiod, an appropriate amount (typically 200–400 mL per tray) of Hyponex (N:P:K=6:10:5, 1:10000 dilution) was given daily.

Growth and senescence

Seedlings were harvested at appropriate intervals, and the lengths of their first leaves measured. Then, pieces of leaf blade (5 mm×5 mm squares) were excised from the center of the upper halves of the leaf blades, placed individually in 2-mL sampling tubes with a metal-bead, quickly frozen, and stored at –60°C. Chlorophyll and soluble-protein contents were measured according to Fukaya *et al.* (2012), except that the samples were powdered with a sample crusher (Multi-beads shocker, Yasui-Kikai Co., Ltd., Osaka, Japan).

Photosynthetic properties

Approximately 15 maize first leaves, with their sheaths individually inserted into water-filled micro-centrifugation tubes, were placed in an assimilation box (175×75×50 mm, L×W×H) equipped with two closable tube fittings and a built-in CO₂ monitor (CO₂-mini, Custom Co., Tokyo, Japan) and put in a water bath set to 25°C. First, the box was filled with high-oxygen air (25% O₂, 500 ppm CO₂, balance N₂), closed, and illuminated with a white LED light (LE-H635, Twinbard Co., Niigata, Japan) 5 cm above the top panel (200 μmol m^{–2} s^{–1}). The CO₂ concentration was recorded every 2 min, and a CO₂ absorption rate (around 400 to 200 ppm) was calculated as the net photosynthesis (Pn). Then, the light was turned off, the box wrapped with aluminum foil, and the CO₂ concentration recorded at 2-min intervals until a constant rate of CO₂ release (dark respiration, Dr) was obtained. Then, the air within the box was replaced with low-oxygen air (5% O₂, 500 ppm CO₂, balance N₂), and the same procedure was repeated. The gross photosynthesis rate (Pg) was obtained by summing Pn and Dr. In this study, the degree of O₂ inhibition was calculated according to the equation:

$$\text{O}_2 \text{ inhibition (\%)} = [(Pg_{(5\%)} - Pg_{(25\%)}) / Pg_{(5\%)}] \times 100$$

where Pg_(5%) and Pg_(25%) represent Pg under a 5% and 25% O₂ atmosphere, respectively.

Microscopic observations

MCs and BSCs were separated according to Kanai and Edwards (1973) with slight modifications. Leaf blades (upper 3/4) were sliced with a razor, perpendicular to the leaf vein, to less than 1 mm in width. Approximately 1 g of leaf slices were immersed in 20 mL of enzyme solution [0.6 M sorbitol, 20 mM HEPES-NaOH (pH 5.5), 5 mM MgCl₂, 2% cellulase YC], vacuum-infiltrated, and incubated for 2.5 h at 25°C with shaking. After the exchange of enzyme solution and additional 2-h incubation, the suspension was filtered successively through a metal mesh with 500-μm pores and a sheet of nylon mesh with 100-μm pores. The filtrate was further filtered through 40-μm nylon mesh, and the filtrate was centrifuged to obtain MC-protoplasts. Meanwhile, the residue on the 100-μm nylon mesh was re-suspended in small volume of TAN buffer [20 mM Tris–HCl (pH 7.6), 0.5 mM EDTA, 7 mM 2-mercaptoethanol, 1.2 mM spermidine, 0.4 mM PMSF] containing 0.5 M sucrose, vortexed briefly, and the suspension was filtered again through a sheet of 100-μm nylon mesh to obtain BSCs as residues retained on that sheet. MCs and BSCs were re-suspended in 3 mL of TAN buffer containing 0.5 M sucrose and used for DAPI-fluorescence microscopy. Samples were mixed on a glass slide with equal volumes of fixative solution (1% glutaraldehyde in TAN buffer) and staining solution (1 μg mL^{–1} DAPI in TAN buffer), and squashed adequately; for observing BSC chloroplasts, squashing was conducted by gentle tapping with the fixed end of tweezers. The DAPI-stained samples were observed under UV irradiation with a fluorescence microscope (BX-60/BX-FLA, Olympus, Tokyo, Japan).

Results

Growth, maturation, and senescence of first leaves

Under our culture conditions, the first leaves emerged around day 3, with the leaf blades and leaf sheaths reaching maximum length on day 5 and day 9, respectively (Fig. 1). The soluble-protein and chlorophyll contents respectively reached maximum values on day 7 and day 9 and then declined. Thus, the first leaves appeared to have finished their growth and maturation by day 9 and then entered the senescence phase.

Changes in photosynthetic properties

Seven-day-old or younger maize leaves did not exhibit O₂ inhibition, as is expected for a typical C₄ plant. However, 9-day-old or older leaves did exhibit O₂ inhibition (Fig. 1), demonstrating a C₃-like photosynthetic property in these senescing leaves.

Fig. 1. Changes in the size, chlorophyll content, soluble-protein content, and the degree of O₂ inhibition during maturation and senescence of the first leaves of maize. Vertical lines represent the SD. The data for O₂ inhibition represent results from a single series of measurements, each of which was conducted with 15 leaves.

The behavior of chloroplast nucleoids

On day 5, nucleoids in MC chloroplasts had already dispersed throughout the whole chloroplast whereas those in BSC chloroplasts were still localized at the periphery (Fig. 2), features often observed in mature and immature chloroplasts, respectively (Miyamura *et al.*

1990). On day 7 and later, the nucleoids in the BSC chloroplasts also migrated to the interior of the organelles. On day 19, MC chloroplasts had almost completely lost their nucleoids, whereas BSC chloroplasts still retained their nucleoids.

Discussion

C₃-like photosynthesis in senescing maize leaves

Our results that maize first leaves that have completed their growth and maturation exhibit O₂ inhibition, a C₃-like photosynthetic property (Fig. 1), is consistent with a previous report that old maize leaves exhibit an elevated CO₂ compensation point, another C₃-like photosynthetic property (Crespo *et al.* 1979). Occurrence of O₂ inhibition, however, provides a more direct indication of insufficient CO₂ pumping, because it means a decreased CO₂/O₂ ratio around Rubisco, whereas an elevated CO₂ compensation point may also result from an increased respiration/photosynthesis ratio (Yoshioka *et al.* 2009).

Whether such C₃-like photosynthetic property occurs also in the second and the following leaves of maize during senescence is presently unclear; it should be examined in a future work. However, we have found that maize coleoptiles do not perform typical C₄ photosynthesis (Fukaya *et al.* 2012). Studies on the occurrence of non-C₄ photosynthesis in C₄ plants may give us clues to understand the advantages and disadvantages of C₄ photosynthesis. For example, decreased CO₂ pumping activity in senescing leaves may not be so problematic, because senescing leaves are generally shaded with the younger new leaves and are thus under low-light conditions. C₄ photosynthesis is not preferable under such

Fig. 2. Changes in the chloroplast-nucleoids in MCs and BSCs during maturation and senescence of the first leaves of maize. Scale bars=5 μm.

conditions, because its light-use efficiency for carbon fixation should be low relative to C_3 photosynthesis, due to its additional load to drive CO_2 pumping. In this context, however, authentic C_3 photosynthesis (instead of C_4 photosynthesis with insufficient CO_2 pumping) appears more profitable in senescing leaves under shaded conditions. Thus, the possibility of direct CO_2 fixation by Rubisco in MC chloroplasts in senescing maize leaves seems worth examining in future work.

Different behaviors of chloroplast nucleoids between MCs and BSCs

Our results are partly consistent with a previous report by Lindbeck *et al.* (1989), in that BSC chloroplasts contained a larger number of nucleoids than MC chloroplasts. However, the nucleoids in BSC chloroplasts did not appear smaller than those in MC chloroplasts, especially during the senescence phase; the number and size of chloroplast-nucleoids declined during senescence in both cell types, but the decline began earlier and proceeded faster in MCs than in BSCs (Fig. 2).

Our observation strongly suggests that MCs senesce earlier than BSCs, as degradation of chloroplast nucleoids is an early marker of senescence (Sodmergen *et al.*

1989, 1991, Inada *et al.* 1999, 2002). We propose two possible explanations for such differential senescence or differential degradation of chloroplast nucleoids between MCs and BSCs. The first is based on the functional difference between MCs and BSCs. In NADP-ME type C_4 plants in which maize is included, MC chloroplasts have an oxygen-evolving function but BSC chloroplasts do not. Therefore, the levels of O_2 and derivative reactive oxygen species (ROS) should be higher in MCs. The higher ROS level may cause more damage to, and thus earlier degradation of, DNA in MC chloroplasts, leading to earlier chloroplast dysfunction and, finally, earlier senescence of MCs. Kumar *et al.* (2015) indeed reported more severe damage to DNA in MC chloroplasts than in BSC chloroplasts. The second explanation presupposes a general rule that senescence within a plant organ progresses towards the vascular bundles (VBs), as reported for rice coleoptiles (Inada *et al.* 2002). Such a rule seems quite reasonable from the viewpoint of recovery and translocation of resources (such as nitrogen and phosphorus) from the senescing organs, and if it is also applicable to maize leaves, MCs distant from VBs should necessarily senesce earlier than BSCs. Which idea is more plausible is presently unclear, but analyses

Fig. 3. Schematic representation of the mechanism underlying C_3 -like photosynthetic properties of senescing maize leaves. In young leaves, MCs and BSCs are both healthy and cooperatively perform C_4 photosynthesis. In senescing leaves, MCs senesce earlier, causing inefficient CO_2 pumping, the insufficient concentration of CO_2 in BSCs, and finally, C_3 -like photosynthetic properties.

on the behavior of MC- and BSC chloroplast nucleoids in NAD-ME type or PEP-CK type C₄ plants, in which both MCs and BSCs have oxygen-evolving chloroplasts, may give us hints.

A hypothesis on the occurrence of C₃-like photosynthesis in senescing maize leaves

Taken together, our results suggest that insufficient CO₂ pumping, resulting from earlier senescence of MCs, may be responsible for the C₃-like photosynthetic properties in senescing maize leaves (Fig. 3). Our observation that CO₂ compensation point increased and PEPC/Rubisco ratio together declined during senescence of maize leaves (unpublished) also supports this opinion. To test this hypothesis, detailed analyses on the progression of senescence and alteration of photosynthetic properties, including both morphological and biochemical ones, will be conducted in near future.

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