The maize coleoptiles do not perform typical C_4 photosynthesis: investigation with special reference to anatomy, photosynthetic property, and gene expression

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Summary: Maize (*Zea mays* L.) is one of the most representative C_4 plants. However, several reports have suggested the occurrence of C_3 photosynthesis in this C_4 plant. We examined the photosynthetic characteristics of the coleoptiles, as well as the first, the second, and the third leaves of maize seedlings at their maturities. All the foliage leaves examined, including the first leaf sheath, exhibited representative Kranz anatomy, low CO_2 compensation point, and expression of genes for enzymes involved in the C_4-dicarboxylic acid cycle. In contrast, coleoptiles showed no Kranz anatomy and extremely high CO_2 compensation point. The expression of C_4-specific genes in the coleoptiles was hampered at various levels. These results strongly suggest that the maize coleoptiles do not perform typical C_4 photosynthesis.

Key words: C_3 photosynthesis, C_4 photosynthesis, CO_2 compensation point, coleoptile, Kranz anatomy, maize

INTRODUCTION

C_4 photosynthesis is characterized by its CO_2-concentrating mechanism, called the C_4 dicarboxylic acid cycle. Almost all C_4 plants have a specialized leaf structure, called Kranz anatomy. Kranz anatomy is composed of two distinct types of cells (mesophyll cells and bundle sheath cells) arranged concentrically around vascular bundles, and plays an important role in the CO_2 concentrating mechanism. In maize, atmospheric CO_2 is primarily fixed by phosphoenolpyruvate carboxylase (PEPC) in mesophyll cells to form oxaloacetate, which is then converted to malate and transported to the bundle sheath cells. The malate is then decarboxylated by NADP-dependent malic enzyme (NADP-ME) present in the bundle sheath cells, and the CO_2 released by the reaction is re-fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the chloroplasts of the bundle sheath cells. The other reaction product of the decarboxylation, pyruvate, is returned to the mesophyll cells and is used to regenerate phosphoenolpyruvate, the primary acceptor of carbon, by the action of pyruvate orthophosphate dikinase (PPDK).

Maize is one of the most well-known C_4 plants. However, several researchers have reported the occurrence of C_3-mode photosynthesis in some organs and tissues of maize. Williams and Kennedy (1976) reported that the photosynthetic characteristics of maize leaves became more C_3-like during leaf senescence. Crespo et al. (1979) reported that the first leaves of maize seedlings exhibited C_3-like photosynthetic characteristics. Nelson and Langdale (1989) reported that, in the husk leaves surrounding the female inflorescence, mesophyll cells located distantly from vascular bundles performed C_3 photosynthesis. They also reported the absence of C_4-cycle related enzymes in the coleoptiles based on the results of immunostaining of tissue sections; however, photosynthetic characteristics, as well as the mode of expression of photosynthesis-related genes, of coleoptiles have not been examined in detail (Langdale et al. 1988).

In the present study, we examined the photosynthetic

characteristics and the mode of expression of photosynthesis-related genes of maize coleoptiles, which are the first-emerging aerial organ that has both protective and photosynthetic functions. Our results strongly supported the non-C_4 mode of photosynthesis in maize coleoptiles. The biological significance of the result is discussed from several viewpoints, such as control of gene expression, developmental/anatomical constraints, and adaptation to the environment.

MATERIALS AND METHODS

Plant material and microscopic observation

Seeds of maize (*Zea mays* cv. Golden Cross Bantam T51, from Ai-san-syubyo Co. Ltd., Aichi, Japan, or Canberra 90, from Sakata Co. Ltd., Yokohama, Japan) were soaked for 18 h in water, and planted in vermiculite packed in Jiffy pots (Sakata Co. Ltd., Yokohama, Japan). Seedlings were grown in a growth chamber at 27°C with a photoperiod of 14 h light and 10 h dark. The photon flux density during the light period was about 170 μmol m^{-2} s^{-1}. Appropriate amounts of nutrients [Hyponex powder (N:P:K = 6.5:6:19) dissolved in deionized water at a 1: 10,000 ratio] were supplied every day. The length of coleoptiles and leaves was measured with a digital caliper. For extraction of chlorophyll, coleoptiles and leaf blades were ground to a powder in liquid nitrogen with a mortar and pestle, suspended in 80% (v/v) acetone, shaken for 5 min in sampling tubes, and then centrifuged. The supernatant was used for spectrophotometric quantification of chlorophyll, according to Porra et al. (1989). For extraction of soluble proteins, coleoptiles and leaf blades were ground to a powder in liquid nitrogen with a mortar and pestle, suspended in protein extraction buffer [10 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)], shaken for 5 min in sampling tubes, and then centrifuged. The supernatant was recovered and used for quantification of protein, using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with γ-globulin as a standard. For microscopic observations of Kranz anatomy, pieces of sample organs were fixed in 1% (w/v) glutaraldehyde and 2% (w/v) formaldehyde dissolved in 100 mM sodium phosphate buffer (pH 7.0), embedded in 5% (w/v) agar, sectioned to 40-60 μm thickness with a micro-slicer (DTK-1500, DSK, Kyoto, Japan), and observed with a microscope (BX-60, Olympus, Tokyo, Japan).

Estimation of photosynthetic characteristics

Light response curves of photosynthesis were drawn using an open-flow system with an infra-red gas analyzer (IRGA; LI-800, LI-COR, Lincoln, NE, USA), as described previously (Yoshioka et al. 2009). The CO_2 compensation points of photosynthetic organs were measured with a closed circulation system equipped with the same IRGA, as described previously (Yoshioka et al. 2009). After measurements of photosynthetic characteristics, sample organs were dried for 3 days at 80°C and weighed.

Western blotting analysis

Soluble proteins were extracted from photosynthetic organs of maize seedlings and quantified as described above. Then, the protein extracts were mixed with 1/10 volumes of 100% (w/v) trichloroacetic acid (TCA) solution, chilled on ice for 30 min and centrifuged for 15 min at 18,500 *g* and 4°C to sediment proteins. The protein pellets were washed with ice-cold 80% (v/v) acetone, dried, dissolved in standard SDS-sample buffer. After boiling for 3 min, the protein samples were centrifuged for 5 min at 18,500 *g* and 25°C, and the supernatant was recovered. Proteins were separated by SDS-PAGE using a Mini-PROTEAN II cell (Bio-Rad, Hercules, CA, USA) and visualized by silver staining (Westmeiner 1997). For western blotting analysis, the separated proteins were blotted onto nitrocellulose- [for detection of Rubisco large subunit (LSU) and C_4-PEPC] or PVDF- (for detection of NADP-ME) membranes using a Mini Trans-Blot apparatus (Bio-Rad, Hercules, CA, USA). Bands of specific proteins (Rubisco LSU, C_4-PEPC, and NADP-ME) were detected using primary antibodies raised against respective target proteins (Ishida et al. 1997, Maurino et al. 1996, Ueno

et al. 2000), secondary antibody (goat anti-rabbit IgG) conjugated with horseradish peroxidase (HRP), and the Supersignal detection kit (Pierce, Rockford, IL, USA) for nitrocellulose membrane or the ECL Plus Western blotting detection kit (GE Healthcare, Buckinghamshire, UK) for PVDF membrane. The anti-NADP-ME antibody recognizes both C_4 (62 kDa) and C_3 (72 kDa) isoforms (Maurino et al .1996), while the anti-PEPC antibody recognizes the C_4-specific isoform only (Ueno et al. 2000).

Total RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR)

Sample organs were immediately frozen in liquid nitrogen and stored at -60°C until use. Dissection of the coleoptiles into the regions surrounding vascular bundles and the regions distant from vascular bundles was done manually under a binocular with a razor blade. For RT-PCR analysis, total RNA was isolated from various organs of the maize seedlings, using PureLink Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA). Contaminating genomic DNA was removed by treatment with DNase I, followed by successive extractions with phenol/chloroform and chloroform, and RNA was precipitated with 2-propanol. RT-PCR was performed using PCR EXPRESS (HYBAID) and RNA PCR Kit (AMV) ver.3.0 (TaKaRa, Otsu, Japan) according to the manufacturers' protocols. The primer sequences and annealing temperatures for the respective primer sets are shown in Table 1. For details, also see the legend to Fig. 5.

Primer sequences for PEPC genes were designed based on the reports by Kawamura et al. (1992) and Hahnen et al. (2003). While expression of C_4-form PEPC is restricted to leaf mesophyll cells under illumination, the C_3-form PEPC is expressed not only in green leaves but also in etiolated leaves and roots (Kawamura et al. 1992). Primer sequences for NADP-ME genes were designed based on the report by Tausta et al. (2002). While expression of C_4-form NADP-ME is restricted to leaf bundle sheath cells, the non-C_4-form NADP-

Table 1 Primer systems applied in this study

Target	Direction	Sequense	Product size (bp)	Annealing temp. (°C)	Reference	Accession No.
C_4-PEPC	Forward Reverse	agaactcaagccctttgggaagc gtcggcgaactccttggacagc	248	60	Hahnen *et al*. 2003	BT040786.1
C_3-PEPC	Forward Reverse	ggaactgcatccattgggtgagaa gagtccatgatctccttggagagg	243	60	Hahnen *et al*. 2003 Kawamura *et al*. 1992	NM_001111968.1
C_4-NADP-ME	Forward Reverse	ggtttgttagcagcactcaag cagggactataaacaacagagtacc	897	55	Tausta *et al*. 2002	NM_001111843
C_3-NADP-ME	Forward Reverse	ggtttgttagcagcactcaag gcgccaaatgttccagtatc	1079	55	Tausta *et al*. 2002	NM_001111913
C_4-PPDK	Forward Reverse	gtcgttgacgccgcgccgatacag ccgtcgacgatctcggcccag	242	65	Hahnen *et al*. 2003	J03901.1
CyPPDKZm1	Forward Reverse	gttgttcagcctagctagctagcgctg cgcccatgtactcctccacccaccgcaggccgtc	305	60	Sheen 1991	
CyPPDKZm2	Forward Reverse	gcccgctccatgtggccgttc gccgtcgaggacctccgccc	235	60	Hahnen *et al*. 2003 Sheen 1991	
rbcS1	Forward Reverse	acggacgacctgctgaagcaggtgg ggtggaaggcgtccgggtaggatttg	226	65	Hahnen *et al*. 2003	NM_001111824
rbcS2	Forward Reverse	ggtgtacaaggagctgcaggaggc ggcagaggcatggccatgggtcg	168	65	Hahnen *et al*. 2003	Y09214.1
rbcS universal	Forward Reverse	cgctgtcgacggacgacctg agccgatgacgcggtggaag	249	65		NM_001111824, Y09214.1
GAPDH	Forward Reverse	ctggtttctaccgacttccttg cggcatacacaagcagcaac	204	55	Hahnen *et al*. 2003	EU953063.1

ME is expressed not only in leaves but also in roots and endosperm (Tausta et al. 2002). Primer sequences for PPDK genes were designed based on the reports by Sheen (1991) and Hahnen et al. (2003). While expression of C_4, chloroplast-localized PPDK (*C4ppdkZm1*) is highly specific for leaf mesophyll cells, the non-C_4, cytoplasmic PPDK genes (*cyppdkZm1* and *cyppdkZm2*) are reported to be constitutively expressed at low levels (Sheen 1991). The *C4ppdkZm1* and *cyppdkZm1* transcripts are derived from the same locus, harboring two overlapping genes (in other words, they are the products of alternative transcription starts and alternative splicing of one gene), while the *cyppdkZm2* is encoded by a distinct locus (Sheen 1991). Primer sequences for genes encoding the small subunit of Rubisco (*rbcS*) were designed based on the report by Hahnen et al. (2003). It is reported that C_3 tissues of maize (husk mesophyll cells distant from vascular bundles) show higher preference for *rbcS1* expression compared to C_4 tissues (Hahnen et al. 2003). The cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was selected as a constitutively expressed gene to be used as an internal standard, as reported by Hahnen et al. (2003).

RESULTS

Growth of maize seedlings and their photosynthetic organs

Figure 1 summarizes the growth and senescence of photosynthetic organs of maize seedlings. The coleoptiles grew rapidly, reached their maximum sizes on day 4, and then became slightly smaller. The chlorophyll content and soluble protein content of the coleoptiles reached their maximum values on day 5 and day 2, respectively, and then declined. The first leaf blades reached their maximum sizes on day 6, while the growth of the leaf sheath continued until day 8. The chlorophyll content of the first leaf blade peaked around day 7, while the soluble protein content already had peaked on day 5, when the leaf blades still continued to grow. Growth of

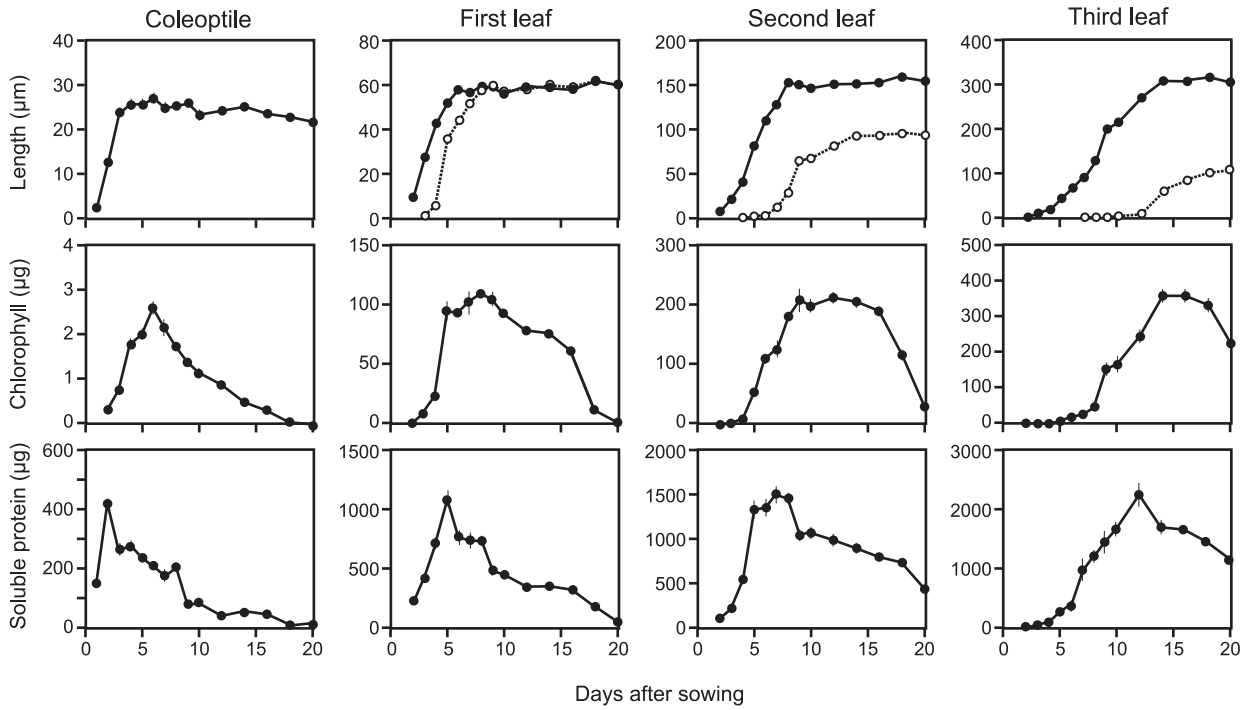

Figure 1
Growth of photosynthetic organs of maize seedlings. Length (top), total chlorophyll content (middle), and total soluble protein content (bottom) of coleoptiles, as well as the first, the second, and the third leaves of maize seedlings were followed for 20 days after sowing. For foliage leaves, length of leaf blades (filled circles) and leaf sheaths (open circles) was measured separately, and total chlorophyll and total protein contents were measured for leaf blades only. Each data point represents the mean from 10 measurements. Vertical bars represent SE.

the second leaf blade had been completed by day 8, while that of the second leaf sheaths continued until day 14. Chlorophyll and soluble protein contents of the second leaf blades reached their maximum values on day 9 and day 7, respectively, and declined after a short plateau. The third leaf blades completed their growth by day 14, while the third leaf sheaths just began to grow on day 12. Chlorophyll and soluble protein contents of the third leaf blades reached their maximum values on day 14 and day 12, respectively, and then declined. Thus, it seems that the photosynthetic organs of maize seedlings first accumulate soluble proteins to their maximum levels, then reach their maximum sizes, and finally accumulate chlorophyll to their maximum levels. Based on these observations, we regarded the timing when chlorophyll content reached the maximum value as the timing of maturities of respective organs. In the following experiments, therefore, we used the coleoptiles, the first, the second, and the third leaves from 6-, 7-, 9-, and 14-day-old seedlings, respectively (Fig. 2A).

Anatomy

The coleoptiles have only two vascular bundles (Fig. 2B). The cells around the vascular bundles and the parenchyma cells near the outer surface of the coleoptiles contained green chloroplasts. The chloroplast-containing cells around the vascular bundles did not show representative Kranz anatomy. In contrast to the case with coleoptiles, the first, the second, and the third leaf blades demonstrated representative Kranz anatomy (Fig. 2C). The first leaf sheaths also exhibited Kranz anatomy, although vascular bundles are sparsely distributed and the parenchyma cells located distantly from the vascular bundles lack chloroplasts. We ignored the second and the

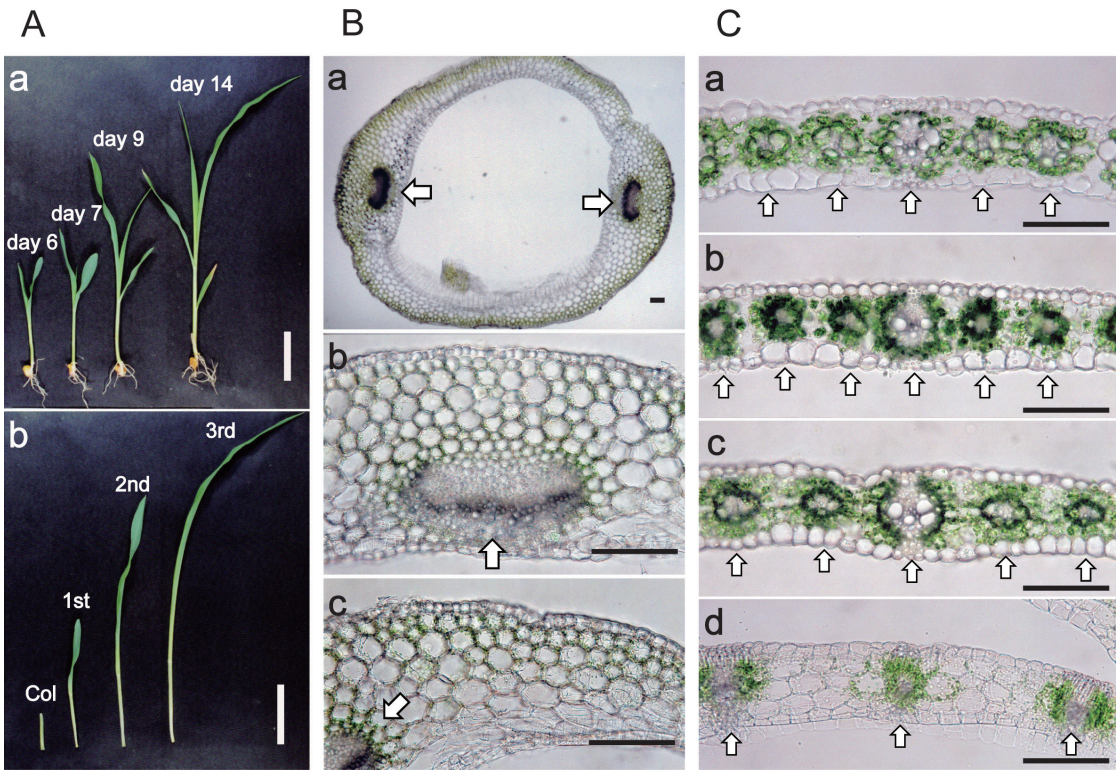

Figure 2
Morphological observations of the maize photosynthetic organs at maturity. **A.** Overview of seedlings (a) and focal photosynthetic organs (b) used for the analyses. Coleoptiles, first leaves, second leaves, and third leaves were harvested 6, 7, 9, and 14 days after sowing, respectively, when their chlorophyll content reached maximum values. **B.** Cross-sections of coleoptiles. Overview (a), as well as close-up of tissues around (b) and distant from (c) vascular bundles are shown. **C.** Cross-sections of foliage leaves. The leaf blades of the first (a), the second (b), and the third (c) leaves, as well as the sheath of the first leaf (d), are shown. Samples were fixed and sectioned to 40-60 μm thickness, and observed by bright-field microscopy. Arrows, vascular bundles. Scale bars represent 5 cm in **A** and 100 μm in **B** and **C**.

third leaf sheaths in the following experiments, because the major part of them was shaded within the tube of the first leaf sheath, resulting in poor development of photosynthetic capacity.

Photosynthetic characteristics

We then examined photosynthetic characteristics of maize organs (Fig. 3). To compare photosynthetic activities between tubular organs (coleoptiles and leaf sheaths) with flat organs (foliage leaf blades) on the same basis, photosynthetic activity is expressed on a dry-weight basis (Fig. 3A), instead of a conventional leaf-area basis. Photosynthesis rates of the foliage leaves were high and did not saturate within the ranges of photon flux densities examined (up to 500 μmol m^{-2} s^{-1}). In contrast, photosynthesis of coleoptiles saturated under low light intensity (about 100 μmol m^{-2} s^{-1}), and their maximum net photosynthesis rates were marginal, close to zero. The first leaf sheaths exhibited a low, but substantial level of photosynthetic activity, and their photosynthetic rate saturated at about 400 μmol m^{-2} s^{-1}.

The first, the second, and the third leaf blades all exhibited quite low (<10 ppm) CO_2 compensation points characteristic of C_4 photosynthesis (Fig. 3B). In contrast, coleoptiles exhibited an extremely high CO_2 compensation point (400-500 ppm). The first leaf sheaths showed quite a low CO_2 compensation point (approximately 10 ppm) characteristic of C_4 photosynthesis, in spite of their relatively low photosynthetic carbon assimilation rates.

Accumulation of proteins involved in C4 dicarboxylic acid cycle

C_4 photosynthesis requires the function of several enzymes involved in the C_4 dicarboxylic acid cycle that concentrates CO_2. Among them, accumulation of C_4-specific isoforms of PEPC and NADP-ME was examined by western blotting analysis. For comparison, accumulation of a Calvin cycle enzyme, Rubisco, was also examined. As shown in Fig. 4, only a trace amount of C_4-specific PEPC was detected in the coleoptiles, whereas the foliage leaf blades contained a large amount of C_4-PEPC protein. Comparison of the signal intensities with a dilution series demonstrated that the level of C_4-specific PEPC in the coleoptiles was 1/10 to 1/30 of that in the first leaf blades. C_4- NADP-ME was detected in the first, the second, and the third leaf blades, but was below the detection limit in the coleoptiles (less than 1/30 of the first leaf blades). In contrast, Rubisco LSU and C_3- NADP-ME accumulated in both foliage leaf blades and coleoptiles to similar levels. Thus, the coleoptiles appeared to be incapable of typical C_4 photosynthesis

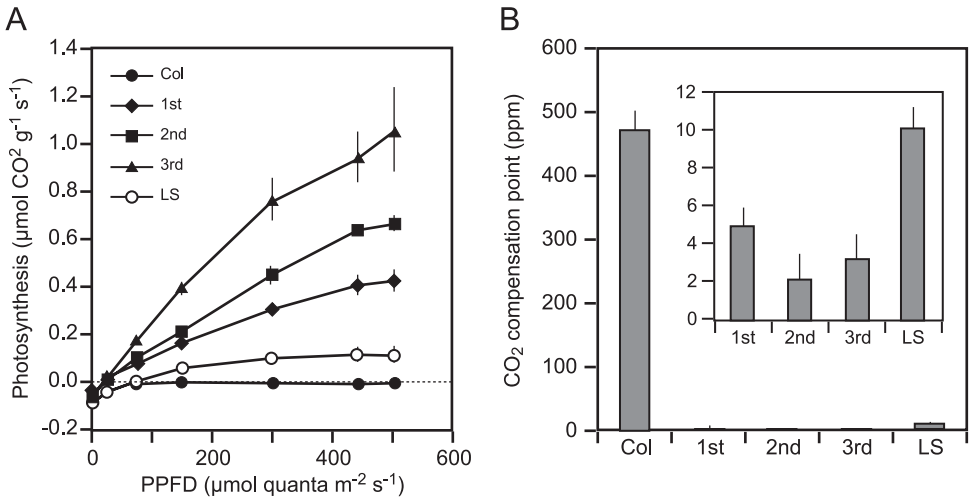

Figure 3

Photosynthetic characteristics of photosynthetic organs of maize seedlings. **A.** Light-response curves. Each data point represents the mean from three measurements. Vertical bars represent SE. **B.** CO_2 compensation points. Each data represents the mean from three to six measurements. Vertical bars represent SE. Col, coleoptiles; 1st, first leaf blades; 2nd, second leaf blades; 3rd, third leaf blades; LS, 1st leaf sheaths. Inset in B shows an expanded graph of the CO_2 compensation points of foliage leaves.

because of the lack of an important enzyme (C_4-NADP-ME), although they contain C_4-PEPC at quite a low level. The first leaf sheaths were found to accumulate C_4-PEPC, C_3- and C_4- NADP-ME and Rubisco LSU proteins to levels comparable to leaf blades (1/3 to the same amount as the first leaf blades), supporting their C_4-photosynthetic nature.

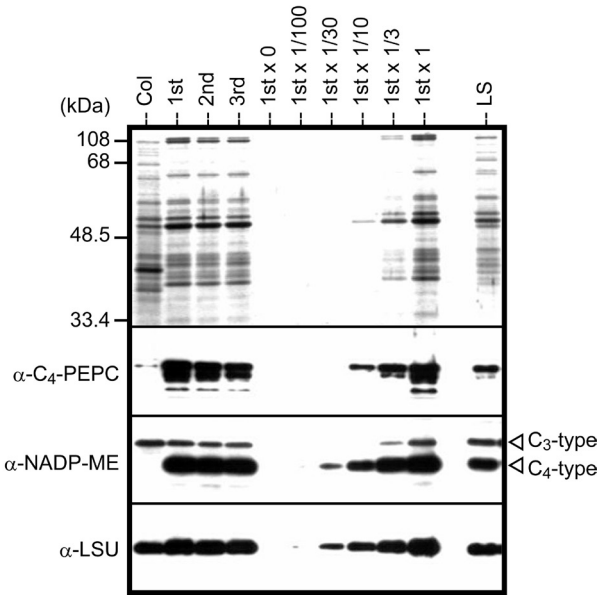

Figure 4
Western blotting analyses of enzymes involved in carbon fixation. Soluble proteins extracted from photosynthetic organs of maize seedlings were separated by SDS-PAGE (each lane except those of the dilution series was loaded with 5 μg of protein), blotted onto nitrocellulose or PVDF membranes, and probed with antibodies raised against C_4-type PEPC, NADP-ME, and Rubisco LSU. For semi-quantitative comparison, a dilution series of proteins obtained from first leaf blades was included in the analyses. Top panel shows silver-stained gel. Numbers to the left of the top panel represent sizes of markers in kDa. Positions of C_3- and C_4-types of NADP-ME are indicated by arrowheads on the right.

Expression of genes for C_4 photosynthesis enzymes and their non-C_4 isoforms

The expression of C_4 photosynthesis-specific enzymes in the coleoptiles was further examined by analyzing their transcript accumulation. The genes examined by RT-PCR included those for enzymes involved in the C_4-dicarboxylic acid cycle, their non-C_4 isoforms, Calvin cycle enzymes, and a constitutively expressed enzyme as an internal control (Table 1). The organs examined included photosynthetic organs of maize seedlings (coleoptiles, the first to the third leaf blades, and the first leaf sheath), husk leaves, primary roots, and embryos in imbibed seeds. In addition, coleoptiles dissected into two parts, i.e., the regions near vascular bundles and the regions that were distant from the vascular bundles, were included in the specimen.

Figure 5 shows representative RT-PCR data selected from three experiments with similar results. Due to as much as 30 cycles of PCR amplification, the band intensity may not reflect the difference in the transcript amounts quantitatively. However, the differences in the band intensity, if detected, should suggest differences in the transcript accumulation among the organs.

Transcript for the C_4-PEPC gene was detected in all the photosynthetic organs examined, including coleoptiles. The level of C_4-PEPC transcript in the coleoptiles appeared to be lower compared to that in the foliage leaf blades, which was confirmed by northern blotting analysis (data not shown). Within a coleoptile, the C_4-PEPC transcript level appeared to be slightly higher in the regions near the vascular bundles than in the regions distant from vascular bundles. While the C_4-PEPC gene appeared to be inactive in non-photosynthetic organs (embryos and roots), the C_3-PEPC gene was constitutively expressed in all the organs tested.

Transcript for the C_4-NADP-ME gene was also detected in all the photosynthetic organs examined. However, its transcript level appeared lower in the coleoptiles than in other photosynthetic organs. Within a coleoptile, the transcript level appeared to be higher near the vascular bundles. Among non-photosynthetic organs, embryos accumulated the C_4-NADP-ME transcript to high level. The C_3-NADP-ME gene was constitutively expressed in all the organs tested.

The level of the C_4-PPDK transcript appeared high in the first, the second, and the third leaf blades, low in the first leaf sheaths and husk leaves, and was below the detection limit in coleoptiles, as well as in non-photosynthetic organs. Transcript for *cyppdkZm1*, which encodes a cytosolic, non-C_4 isoform of PPDK, was highly specific for the embryos. However, quite a low

level of *cyppdkZm1* transcript was also detected in the regions near the vascular bundles of coleoptiles. We also examined the expression of the *cyppdkZm2* gene, which encodes another cytosolic, non-C_4 isoform of PPDK, but failed to detect the transcript in any of the organs tested (data not shown).

Transcript for *rbcS* genes was detected in all the photosynthetic organs tested at similar levels. Because no qualitative difference in the mode of gene expression was found between *rbcS1* and *rbcS2* genes (data not shown), only the result with the universal primers, which can amplify both *rbcS1* and *rbcS2* transcripts simultaneously, is shown. Transcript for the GAPDH gene, selected as a constitutively expressed internal control, was equally detected in all the organs examined.

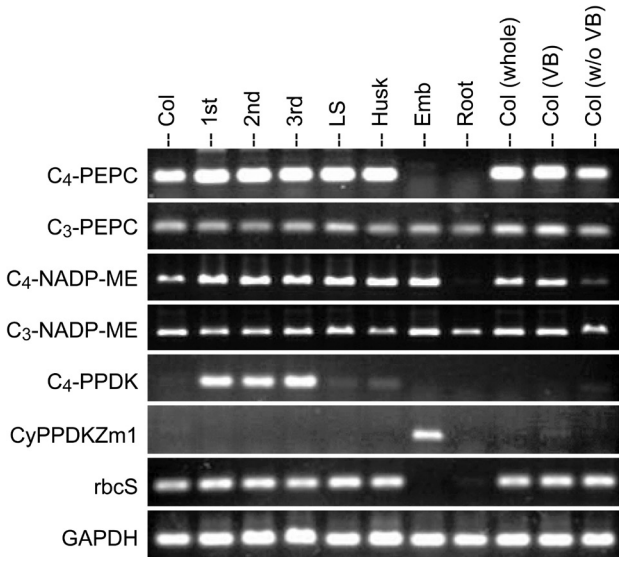

Figure 5
RT-PCR analyses of expression of genes for enzymes involved in the Calvin cycle and C_4 dicarboxylic acid cycle and their non-C_4 isozymes. For each gene, cDNA derived from 100 ng of total RNA with an oligo-dT primer was amplified by PCR. Amplification conditions were 94°C for 2 min and 30 cycles, each cycle at 94°C for 30 sec, at annealing temperatures suitable for the respective primers (Table 1) for 30 sec, and 72°C for 1 min, followed by incubation at 72°C for 10 min. Each lane was loaded with RT-PCR products derived from 15 (for PEPCs, rbcS, and GAPDH) or 30 (for MEs and PPDKs) ng of template RNA. Col and Col (whole), coleoptiles; 1st, first leaf blades; 2nd, second leaf blades; 3rd, third leaf blades; LS, first leaf sheaths; Husk, husk leaves; Emb, Embryos; Root, primary roots; Col (VB), coleoptile parts near vascular bundles; Col (w/o VB), coleoptile parts distant from vascular bundles.

DISCUSSION

Maize coleoptiles do not perform typical C_4 photosynthesis

The maize coleoptiles lacked Kranz anatomy (Fig. 2), exhibited a low light saturation point and high CO_2 compensation point of photosynthesis (Fig. 3), and did not show full expression of enzymes involved in C_4 dicarboxylic acid cycle (Figs. 4 and 5). In general, C_4 plants have Kranz anatomy, exhibit high light saturation point and low CO_2 compensation point of photosynthesis (Edwards and Walker 1983), and show expression of complete set of C_4 cycle-related enzyme genes. Anatomy, photosynthetic characteristics, and the mode of gene expression of maize coleoptiles all suggest that the maize coleoptiles do not perform typical C_4 photosynthesis. In contrast to the case with coleoptiles, the first leaf sheaths showed Kranz anatomy (Fig. 2C), exhibited quite a low CO_2 compensation point (Fig. 3B), and showed a considerable level of expression of a complete set of C_4 cycle genes (Figs. 4 and 5). These results support their C_4-mode of photosynthesis. We also confirmed that the first leaf blades, which were previously reported to perform C_3-like photosynthesis (Crespo et al. 1979), also exhibited characteristics typical for C_4 photosynthesis. Thus, we conclude that the coleoptile is the only photosynthetic organ that does not perform typical C_4 photosynthesis in maize seedlings.

The final definition on the photosynthetic mode of maize coleoptiles, however, awaits further investigations. Because there are several Kranz-less C_4 plants, such as *Hydrilla verticilliata* (Magnin et al. 1997) and *Borszczowia aralocaspica* (Voznesenskaya et al. 2001), the lack of Kranz anatomy (Fig. 2B) does not guarantee the absence of C_4 photosynthesis. The low light saturation point (Fig. 3A) may be adaptation to low-light environment surrounding coleoptiles. The CO_2 compensation point of maize coleoptiles was extraordinarily high (400-500 ppm, Fig. 3B) and may not be so informative in discriminating between C_4 and C_3 photosynthesis: In general, CO_2 compensation point of C_4 plants is <10 ppm, while that of C_3 plants is about 50 to 70 ppm. The extraordinarily high CO_2 compensation point of

coleoptiles is probably attributable to the high number of non-photosynthetic cells in this organ (Fig. 2B), because presence of large amount of non-photosynthetic cells within a photosynthetic organ can result in high CO_2 compensation point of the organ (Yoshioka et al. 2009). The absence of full expression of enzymes involved in C_4 dicarboxylic acid cycle (Figs. 4 and 5) provides more reliable support for the non-C_4 mode of photosynthesis in maize coleoptiles. However, it is believed that C_4 enzymes have evolved via the recruitment of non-C_4, pre-existing enzymatic activities (Tausta et al. 2002), and the non-C_4 isoenzymes, still present in the C_4 plants, may constitute a C_4-like metabolic pathway. Indeed, Hibberd and Quick (2002) reported that the photosynthetic cells within vein tissues in typical C_3 plants contained high activities of (non-C_4) decarboxylation enzymes and performed C_4-like metabolism, i.e., they obtained CO_2 via decarboxylation of organic acids derived from xylem. A similar metabolic pathway may also operate in the photosynthetic cells around the two vascular bundles in the maize coleoptiles. Nonetheless, it seems unlikely that the peripheral photosynthetic cells that are located far away from the vascular bundles are engaged in such C_4-like metabolism. It is possible that photosynthetic properties are different between the cells surrounding the vascular bundles and those on the periphery. Clearly, further analyses on the photosynthetic properties, the expression profile of C_4-cycle enzymes within a coleoptile, and the behavior of photosynthetically fixed carbon, are necessary to clarify the actual mode of photosynthesis in maize coleoptiles.

Possible explanations for the absence of typical C_4 photosynthesis in maize coleoptiles

The reason why the maize coleoptiles do not perform typical C_4 photosynthesis can be examined from several points of view. First of all, the coleoptiles are not the organ specialized for photosynthesis. Their primary role seems to be the protection of the immature leaves within. However, the first leaf sheaths perform C_4 synthesis, although it seems that their primary role is also the protection of immature leaves (and the mechanical support of the leaf blades), rather than photosynthesis. Thus, there should be additional reason(s) why maize coleoptiles do not perform typical C_4 photosynthesis.

Anatomically, coleoptiles have only two vascular bundles, separated far away on the opposite sides of the organ (Fig. 2B). C_4 photosynthesis requires close positioning of mesophyll cells and bundle sheath cells for efficient exchange of metabolites. In maize foliage leaf blades that perform C_4 photosynthesis, the vascular bundles are separated by only two mesophyll cells, and all the cells between vascular bundles are engaged in photosynthesis (Dengler and Nelson 1999). In leaf sheaths that also perform C_4-photosynthesis, the distance between vascular bundles becomes larger; photosynthetic cells are present only around the vascular bundles, and two to three parenchyma cells distant from either vascular bundle lack chloroplasts (Fig. 2C). In husk leaves, which have at least 10 mesophyll cells between vascular bundles (Antonielli and Venanzi 1979), mesophyll cells that are distant from vascular bundles reportedly perform C_3 photosynthesis (Langdale et al. 1988). Thus, it seems that the maize photosynthetic organ becomes more C_3-like as the spacing between vascular bundles becomes larger. Probably, the number of vascular bundles in maize coleoptiles is too small (only 2) and the number of the cells between the vascular bundles is too large (about 60; Fig. 2B), to adopt a C_4-mode of photosynthesis. The report that vein (vascular bundle) spacing influences the photosynthetic gene expression and photosynthetic mode (Langdale et al. 1988) is consistent with this opinion.

From an eco-physiological viewpoint, it is generally accepted that C_4 photosynthesis is beneficial under conditions such as high light, moderate aridity, and low CO_2 concentration. However, coleoptiles grow through soil matrix; thus, they essentially grow under relatively dark, wet and CO_2-rich conditions throughout most of their short lives. In addition, the coleoptiles mostly consist of non-photosynthetic parenchyma cells, and enclose young, photosynthetically incompetent immature leaves within them. Thus, the photosynthetic cells in the coleoptiles might be supplied with enough CO_2 from respiration of these non-photosynthetic cells and (usually

CO_2–rich) soil air (Larcher 2003). C_4 photosynthesis does not seem beneficial under those environmental conditions surrounding coleoptiles, which may partly explain why maize coleoptiles have not employed a typical C_4 photosynthesis. In contrast, the cotyledons of C_4 dicots (such as *Flaveria trinervia* and *Amaranthus hypochondriacus*) perform C_4 photosynthesis (Shu et al. 1999), probably because they are more similar to foliage leaves and may readily experience high light, dryness, and limited CO_2 supply when they expand in the air above ground.

C_4 photosynthesis requires coordinated expression of C_4-specific genes and, in many cases, differentiation of bundle sheath cells and mesophyll cells, leading to the development of Kranz anatomy. We found that the C_4-specific genes were partially active in the maize coleoptiles. However, their expression was hampered at various levels. While expression of C_4-PPDK was repressed strongly at the transcriptional level (Fig. 5), that of the C_4-form of NADP-ME seemed to be repressed substantially at the transcriptional level (Fig. 5) and almost completely at the translational and/or post-translational level(s) (Fig. 4). The expression level of C_4-PEPC was slightly lowered at the transcriptional step (Fig. 5) and further lowered at the translational/post-translational steps. In contrast to general belief (e.g., Offermann et al. 2008), however, a trace amount of C_4-PEPC protein was still detected in the coleoptiles by western blotting analysis (Fig. 4). Moreover, although the coleoptiles lack representative Kranz anatomy (Fig. 2B), the spatial pattern of expression of C_4-specific genes in the coleoptiles seemed to follow a general rule; the expression of C_4-PEPC and C_4-NADP-ME seemed higher in the region near vascular bundles (Fig. 5). These observations suggest that the expression of individual C_4-specific genes is controlled differently according to the positions within a coleoptile at various steps of gene expression. Thus, the coleoptiles may provide a good opportunity to analyze the mechanisms underlying differential expression of C_4 photosynthesis genes, as well as the relationship between gene regulation and organ structures.

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