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Short Communication

Chloroplasts in seeds and dark-grown seedlings of lotus

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Summary

In most higher plants, mature dry seeds have no chloroplasts but etioplasts. Here we show that in a hydrophyte, lotus (*Nelumbo nucifera*), young chloroplasts already exist in shoots of mature dry seeds and that they give rise to mature chloroplasts during germination, even in darkness. These shoots contain chlorophyll and chlorophyll-binding proteins CP1 and LHCP. The unique features of chloroplast formation in *N. nucifera* suggest a unique adaptive strategy for seedling development correlated with the plant's habitat.

Key words: Chloroplast – hypoxia – lotus (*Nelumbo nucifera*) – photosynthesis – seed

Abbreviations: Chl = chlorophyll. – CP = chlorophyll-binding protein. – LHCP = light-harvesting chlorophyll *a/b*-binding protein

Introduction

In most higher plants, mature dry seeds contain etioplasts. After germination seedlings acquire sunlight and oxygen, and etioplasts are changed into mature chloroplasts to start photosynthesis. In hydrophytes, however, seeds germinate under water where the oxygen concentration is low. Since the conversion of etioplasts to chloroplasts needs oxygen-dependent ATP synthesis (Ushimaru et al. 1992), hydrophytes would not be able to initiate photosynthesis if chloroplast construction were required. We recently found that the hydrophyte lotus (*Nelumbo nucifera*) is able to overcome this problem. Lotus seedlings that germinated in darkness produced green shoots (Ushimaru et al. 2001). Here we show that in this species mature dry seeds contain young chloroplasts, which developed into mature chloroplasts during germination under water, even in darkness. These unique features appear to be correlated with the habitat of this species.

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Materials and Methods

Mature seeds of *Nelumbo nucifera* Gaertn. were collected from plants grown in ponds in the Botanical Garden of Kyoto University, Japan. Seeds were germinated at 30 °C in darkness under water or in air. When light-grown seedlings were used, seedlings were germinated

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under fluorescent illumination at an intensity of 140 mmol m^{-2} s^{-1} at 400 to 700 nm (Ushimaru et al. 2001). All manipulations using dark-grown seedlings were performed under a dim green safelight.

For protein extraction, three shoots were homogenized with a glass homogenizer in four volumes (v/w) of an ice-cold homogenizing buffer [50 mmol/L potassium phosphate (pH 7.8), 0.1 % Triton X–100 and 0.1 mmol/L EDTA]. The extracts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 13.7 % acrylamide gels followed by Western blot analysis as reported earlier (Ushimaru et al. 1995). Antibodies raised against chlorophyll-binding protein (CP) 1, light-harvesting chlorophyll *a*/*b*-binding protein (LHCP) (Tanaka et al. 1994), and cytochrome *c* (Ushimaru et al. 1995) were used for western blots. Chlorophyll (Chl) was extracted from shoots and measured according to Mackinney (1941).

For electron microscopy, the green shoots were fixed by immersion in a mixture of 2 % glutaraldehyde and 2 % paraformaldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, for 2 h at 4 °C and postfixed with 1 % osmium tetroxide in the same buffer for 2 h at 4 °C. They were dehydrated through a graded ethanol series and embedded in Spurr-resin. Pale gold ultrathin sections were cut with a Reichert Ultracut-E (Reichert-Tung, Vienna, Austria) mounted on nickel grids. After the grids sections were stained with 1 % potassium permanganate, they were observed with a Hitachi 7500 electron microscope at an accelerating potential of 80 kV.

Figure 1. Seeds and seedlings of lotus (*N. nucifera*). (A) A mature dry seed containing a green shoot. To soften seeds for dissection, imbibition was done at 4 °C (at which germination is prevented) in darkness for 24 h. Bar, 1 cm. (B) Seedlings germinated at 30 °C in the dark or light under water for 7 days (bar, 10 cm). Seedling size showed considerable variation.

Results and Discussion

We recently found that the upper region of shoots from *N. nucifera* seedlings is green when germinated under water and even when germinated in total darkness (Ushimaru et al. 2001) (see also Fig. 1 B, under water, dark), although most higher plants cannot construct chloroplasts in darkness. Surprisingly, in lotus, green shoots were already present in seeds (Fig. 1 A).

We observed chloroplast-like green granules in cells of green shoots under light microscopy (data not shown). To confirm the presence of chloroplasts, we took two approaches: electron microscopic observation and biochemical analysis of chloroplast marker components. As shown in Figure 2 A, young chloroplasts in seeds were round, but those in dark-grown seedlings showed an oval and slightly angular shape typical for ongoing maturation. The most prominent feature of chloroplasts in seeds is that many thylakoids were densely paralleled with each other and contained large starch granules. In these thylakoids, neither grana stacks nor partitions can be seen. However, chloroplasts in dark-grown seedlings (Fig. 2 B) had already well-developed grana as found in light-grown seedlings (Fig. 2 C), though thylakoids were less dense in the former than in the latter. Only small starch granules were scattered among thylakoids in dark- and light-grown seedlings.

In addition to Chl *a* and *b*, Chl-binding proteins CP1 and LHCP were present in green shoots of seeds and dark-grown seedlings (Fig. 3 A and B). These results are consistent with those obtained by electron microscopy. The presence of chloroplast components in dark-grown seedlings is unusual, because chlorophyll is broken down during dark incubation in most plants tissues. Consistent with a low ratio of Chl *b*/Chl *a* (Fig. 2 A), LHCP was relatively low as compared with CP1, which binds only Chl *a*, in shoots in seeds and in dark-grown seedlings (Fig. 2 B). In the light-grown control, however, the ratio of LHCP to CP1 was higher than in dark-grown seedlings, suggesting the development of photosynthetic system in the former seedlings. When light was available, whole shoots of seedlings turned green, even when under hypoxia (Fig. 1 B, under water, light).

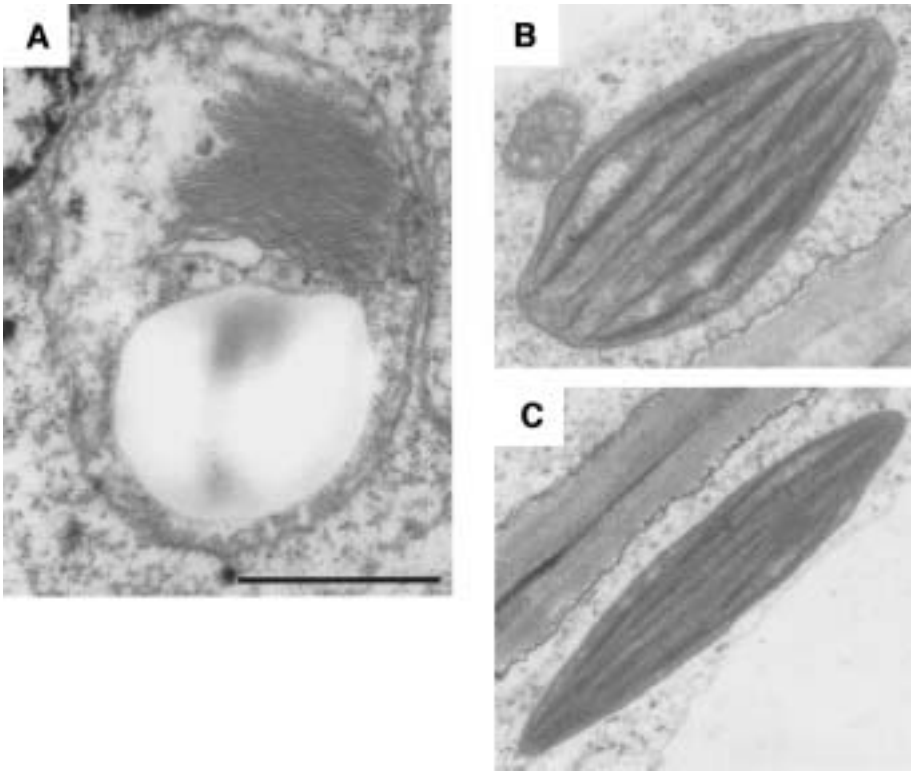

Figure 2. Electron micrographs showing chloroplasts in green shoots dissected from dry seeds (A), 5-day-old dark-grown seedlings (B), and 5-day-old light-grown seedlings (C). Germination was performed aerobically (B and C). Bar, 1.0 μm.

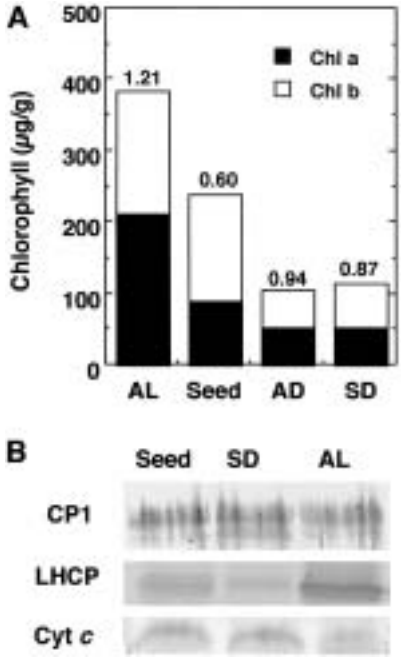

Figure 3. (A) Chlorophyll (Chl) content (μg/g fresh weight) in green regions of shoots. AD, air, dark-grown seedlings; AL, air, light-grown seedlings; SD, submerged (under water), dark-grown seedlings. Numbers above columns represent ratios of Chl *b*/Chl *a*. (B) Western blot of chloroplast proteins in green regions of shoots. Cytochrome (Cyt) *c* is used as a mitochondrial marker.

These unique features of *N. nucifera* are correlated with its habitat. When seeds are germinated under hypoxia, if illuminated, chloroplasts promptly start photosynthesis producing ATP and oxygen. The latter may provide conditions favorable to mitochondrial development. In this species, chloroplasts may have formed and become functional before light is blocked by the thickening of the seed coat. Accumulated starch granules in chloroplasts in shoots of seeds support this idea. However, these features are not common to all hydrophytes, since other hydrophyte species, including rice (*Oryza sativa cv.*) and water chestnut (*Trapa japonica*) have no such features (Ushimaru et al. 1992 and data not shown). Further study is necessary to identify the mechanisms by which Chl is maintained during seed maturation and after germination in darkness in *N. nucifera*.

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