Removal of Magnesium by Mg-dechelatase Is a Major Step in the Chlorophyll-Degrading Pathway in *Ginkgo biloba* in the Process of Autumnal Tints

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Autumnal tints are one of the most manifest and fascinating natural phenomena, but the mechanism of chlorophyll (Chl)-breakdown in deciduous trees has not been fully elucidated. In this study, we analyzed the composition of Chl-related compounds and determined the activities of initial Chl-degrading enzymes in *Ginkgo* leaves at various stages in the process of autumnal coloring. Only pheophytin *a* (Pheo *a*, Mg-free Chl *a*) was detected in yellow leaves by HPLC analysis, and the activity of Mg-dechelatase in yellow leaves was found to be higher than in green leaves. These findings showed that the removal of magnesium from Chl *a* occurred in advance of dephytylation in the *Ginkgo*.

Introduction

The loss of green color in plants during leaf senescence, fruit ripening, and autumnal tinting is due to chlorophyll (Chl) degradation in the chloroplasts. Many efforts have been made to understand this phenomenon at a molecular level, but only few findings have been obtained that elucidate the mechanism of Chl degradation (Matile and Hörtensteiner, 1999). Structural analysis of Chl catabolites has enabled researchers to speculate that the Chl-degrading pathway essentially comprises three steps (Matile and Hörtensteiner, 1999). In the first step, chlorophyllase (Chlase, E. C. 3.1.1.14) catalyzes the hydrolysis of phytol ester of Chl *a* to produce chlorophyllide *a* (Chlide *a*). In the second step, Mg-dechelatase converts Chlide *a* into pheophorbide *a* (Pheide *a*) by dechelating the central Mg-atom. In the third step, Pheide *a* is further degraded by unidentified oxidation enzymes (Matile and Hörtensteiner, 1999; Kräutler and Matile, 1999; Hörtensteiner *et al.*, 1998; Janave, 1997). To our knowledge, this pathway has been mainly investigated in senescent plants or ripening fruits, but few studies have been done on autumnal coloring of the foliage of deciduous trees.

Chl-breakdown is of obvious ecological and economic importance (Kräutler and Matile, 1999). In the present study, we first analyzed the composition of Chl-related compounds, then measured the activities of early Chl-degrading enzymes in *Ginkgo* leaves at various stages in the process of autumnal coloring. Only pheophytin *a* (Pheo *a*, Mg-free Chl *a*) was detected in yellow leaves by HPLC analysis, and the activity of Mg-dechelatase in yellow leaves was found to be higher than in green leaves. These findings show that the removal of magnesium from Chl *a* occurrs in advance of dephytylation in *Ginkgo*. We conclude that Mg-dechelatase plays an important role in the process of autumnal coloring of the foliage of *Ginkgo biloba*.

Material and Methods

Composition analysis

Ginkgo biloba leaves were collected on the campus of Osaka University in the green (Sep. '99), yellow-green (Oct. '99), and yellow (Oct. '99) stages. Pigments were extracted from the fresh leaves with 100% methanol, and the Chl contents determined using their absorption coefficients at 652 and 665 nm (Lichtenthaler, 1987). The separa-

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tion of pigments was carried out by HPLC. Chromatography of pigments was run on a Hitachi HPLC instrument with an L-6200 intelligent pump, L-7300 column oven, and D-2800 chromato-integrator. Fluorescence detection was performed by exciting the pigments at 430 nm and measuring emissions at 667 nm using a SHIMADZU RF-550 spectrofluorometric detector. The sample was quantitatively loaded into a 5 μm Intersil ODS column (4.6 i.d. × 250 mm, GL Sciences, Tokyo, Japan) and eluted by two solvents isocratically--A, 100% methanol for 0–15 min, and B, 50/50 (v/v) methanol/ethyl acetate for 15–25 min at a flow rate of 0.8 ml/min. Each peak was identified by comparing its retention time with that of the corresponding authentic Chl-derivative.

Enzyme assays

Mg-dechelatase and Chlase activities were measured in the leaves harvested at the different stages mentioned above. The enzymes were extracted using a conventional method (Janave, 1997; Mínguez-Mosquera *et al.*, 1994). *Ginkgo* leaves (100 g fr. wt.) were homogenized in chilled acetone (−20 °C) by Polytron (KINEMATICA, Littau-Lucerne, Switzerland). The homogenate was filtered and rinsed with chilled acetone until the filtrate became colorless. The resulting acetone powder was dried at room temperature. The acetone powder was dissolved in a 20 mM sodium phosphate buffer (pH 7.0) containing 0.24% Triton X-100 (1 g acetone powder/30 ml buffer). The solution was centrifuged at 15,000×*g* for 20 min, and the supernatant used for enzymatic assays. The assay method for Mg-dechelatase was conducted by using Mg-chlorin (chlorophyllin) as a substrate (Vicentini and Matile, 1995). The enzyme solution (200 μl, 2 μg/μl protein) was mixed with Mg-chlorin (64 nM final conc.) and incubated at 30 °C for 30 min. The maximum absorbance of Mg-chlorin affords bathochromic shifts from 642 to 686 nm upon removal of the central Mg-atom from the porphyrin ring. Activity was expressed in terms of increment of absorption at 686 nm (Δ686 nm). Chlase activity was measured using Chl *a* as a substrate (Mínguez-Mosquera *et al.*, 1994). The enzyme solution (100 μl) was mixed with an aliquot of an acetone solution of Chl *a* (0.1 mM final conc.) and incubated at 30 °C for 30 min. The reaction was stopped by adding 660 μl of an acetone/n-hexane mixture (2/3, v/v). The mixture was vigorously shaken and then centrifuged at 15,000×*g* for 15 min to separate the organic and aqueous phases. Chl *a* was partitioned into the organic phase, and Chlide *a* remained in the aqueous phase. The absorption at each phase was measured at 430 nm on a SHIMADZU UV-VIS spectrophotometer (UV-1600) and the activity was expressed by the Chlide produced in the aqueous phase.

Results and Discussion

The contents of Chl *a* and *b* decreased dramatically with the progress of autumnal yellowing from 31.3 μg/cm^2 in the green leaves to 1.6 μg/cm^2 in the yellow leaves (Fig. 1). Thus, the amount of Chls in the yellow leaves was only 5% of the amount of green leaves, which confirms that the Chls were degraded during the one-month period of the autumnal coloring process. Although, as previously reported, Chlide *a* and Pheide *a* had been detected in senescent leaves and ripening fruits (Matile and Hörtensteiner, 1999), our experiment showed that only Chl *a*, Chl *b*, and pheophytin *a* (Pheo *a*, magnesium free Chl *a*) were detectable. Other Chl-related catabolites were not found in yellowing *Ginkgo* leaves (Fig. 2). Additionally, HPCL analysis showed that the ratio of the peak area of Pheo *a* to that of Chl *a* increased during the Chl-degrading process.

As shown in Fig. 3, the Mg-dechelatase activity was observed in the three aging stages of the leaves and increased in the leaves at the yellow-

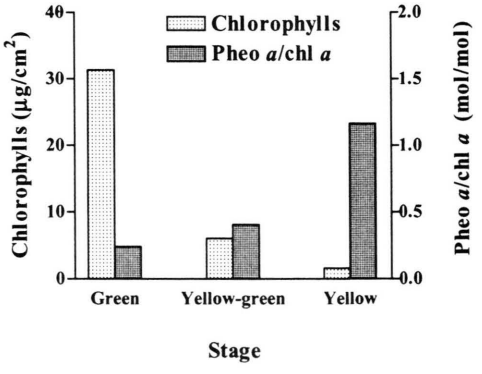

Fig. 1. Changes of Chl contents in *Gingko* leaves during the yellowing process.

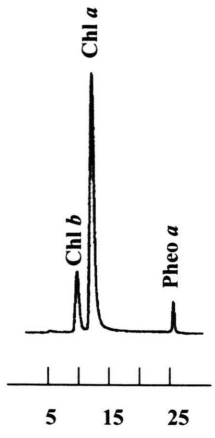

Fig. 2. HPLC profile of Chl-derivatives in yellow leaves of *Ginkgo*.
Separation condition: column, ODS (250 × 4.6 mm); solvent, methanol 100% for 15 min, methanol/ethyl acetate (1:1 v/v) for 10 min; flow rate, 0.8 ml/min; detection, fluorescence (excitation, 430 nm; emission, 667 nm).

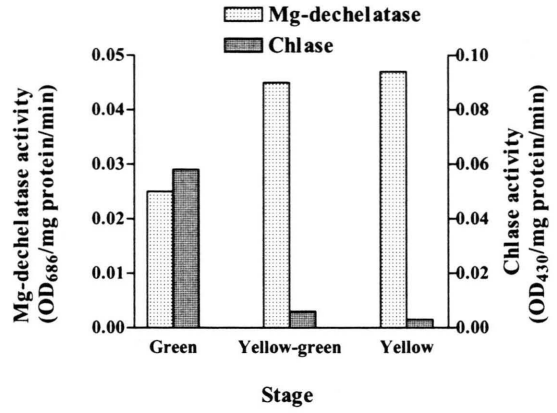

Fig. 3. Changes of Mg-dechelatase and Chlase activities during the yellowing process.
Reaction was carried out at 30 °C for 30 min. In the control experiment, the reaction buffer was used instead of the enzyme solution. All values were corrected by the control value.

green and yellow stages. However, the Chlase activity dramatically decreased.

Chlase is thought to play a critical role in the initial steps of Chl degradation because most of the catabolic products were found to have dephytylated structures (Matile and Hörtensteiner, 1999). Recently, Chlase has been cloned in *Chenopodium album*, *Arabidopsis thaliana* (Tsuchiya *et al.*, 1999) and *Citrus sinesis* (Jacob-Wilk *et al.*, 1999). The relationship between the expression pattern of Chlase mRNAs and leaf-senescence and fruit ripening is now being elucidated.

This study indicated that the activity of Mg-dechelatase increased while the activity of Chlase decreased during yellowing, and that only Pheo *a* was detected as a Chl *a* catabolite in the yellowing *Gingko* leaves. These findings suggest that Mg-dechelatase catalyzes the first step of Chl-degradation during the process of autumnal coloring. Because *Gingko biloba* is a deciduous tree, the recovery of magnesium from the autumnal leaves by the tree is a very important physiological event. Thus, it is reasonable that Mg-dechelatase is more active in the yellow leaves than in green leaves. To elucidate the mechanism of autumnal tinting, further study is needed at the molecular level to analyze the relationship between the expression pattern and the activity of these enzymes. To our knowledge, the present study is the first report that deals with the autumnal tints in relation to Chl-degrading enzymes. Purification and cloning of these Chl-degrading enzymes is in progress in our laboratory.

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