Expression of Chlorophyllase Is Not Induced during Autumnal Yellowing in *Ginkgo biloba*

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Autumnal tints are one of the most fascinating natural phenomena, but the molecular mechanism of chlorophyll (Chl-)degradation in deciduous trees has not been fully understood. In this study, from the leaves of *Ginkgo biloba*, chlorophyllase-homologous *GbCLH* was cloned by RT-PCR with degenerated primers. The expression of *GbCLH* in different yellowing stages was analyzed by Northern hybridization. The expression level of *GbCLH* was highest in green leaves and significantly declined during the process of leaf yellowing. These results suggested that *GbCLH* should be involved in chlorophyll-homeostasis in *Ginkgo biloba*.

Key words: Chlorophyllase, *Ginkgo biloba*, Chlorophyll Degradation

Introduction

It is obvious that chlorophyll (Chl-)degradation is involved in the autumnal coloration of foliage in deciduous trees. However, few studies have been reported on the autumnal coloration of deciduous trees (for review see Matile, 2000). Chl-degradation has been investigated mainly in the senescing leaves of annual grasses and ripening fruits (for review see Hörtensteiner, 1999; Kräutler and Matile, 1999; Matile *et al.*, 1996, 1999; Takamiya *et al.*, 2000). A pathway of Chl-degradation has been deduced by structural elucidation and biochemical analyses of Chl-catabolites. Most Chl-catabolites have no phytyl side-chain, which indicates the importance of hydrolysis of the Chls into chlorophyllides (Chlides) and phytol. This hydrolysis is catalyzed by chlorophyllase (Chlase, E. C. 3.1.1.14), and this process is thought to be the initial step of the Chl-degradation pathway (Holden, 1961). In many plants, such as barley, citrus, sunflower, mango fruit, apple, banana, canora seed, and broccoli floret, the increase of Chlase activity is correlated with the loss of Chl (Gong and Mattheis, 2003; Johnson-Flanagan and McLachlan, 1990; Ketsa *et al.*, 1998; Looney and Patterson, 1967; Purohit, 1982; Rhodes and Wooltoroton, 1967; Rodríguez *et al.*, 1987; Shimokawa, 1981; Trebitsh *et al.*, 1993). Contrary, the activity of Chlase decreased in parallel with the loss of Chls in the leaves of soybean (Majumdar *et al.*, 1991) or the activity of Chlase and the amount of Chls showed no correlation in *Arabidopsis thaliana* (Todorov *et al.*, 2003). This paradox was found even in the same species but different varieties of olive fruits (Roca and Mínguez-Mosquera, 2003) and in the same family but different species of Cruciferae, *Brassica napus* and *Brassica juncea* (Johnson-Flanagan and Spencer, 1996). Chlase activity has been detected not only in senescing leaves or ripening fruits but also in normal green leaves or developing fruit, indicating that Chlase plays an important role in Chl-homeostasis (Mínguez-Mosquera and Gallardo-Guerrero, 1996; Todorov *et al.*, 2003). Furthermore, the expression level pattern of the Chlase gene is not correlated with either leaf senescence or fruit ripening processes. However, some Chlase genes are expressed after the treatment of phytohormones such as methyl jasmonate (MeJA) and ethylene which accelerate the leaf senescence and fruit ripening, respectively (Jacob-Wilk *et al.*, 1999; Tsuchiya *et al.*, 1999). Accordingly, this conflicting evidence led us examine the role of Chlase in the autumnal coloration of deciduous trees, another major Chl-degrading phenomenon.

Abbreviations: Chl, Chlorophyll; Chlide, chlorophyllide; Chlase, chlorophyllase; MeJA, methyl jasmonate.

In this study, we used *Ginkgo biloba* to investigate the relationship between Chlase and the loss of Chl during autumnal coloration. *G. biloba* is an early diverging gymnosperm which has been studied as an unique evolutionary predecessor to angiosperm. In our previous report, pheophytin *a*, the Chl *a*-derivative bearing the phytyl ester sidechain but without chelated Mg^{2+}, was found in the yellowing leaves of *G. biloba.* The Chlase activity significantly decreased with the loss of Chl. In contrast, the activity of Mg-dechelatase markedly increased in the yellowing leaves (Tang *et al.*, 2000). In this report, the cDNA of Chlase of *G. biloba* (*GbCLH*) was cloned and the expression level was analyzed during the yellowing of the leaves. Our experiment clearly showed that the expression level of *GbCLH* significantly decreased in the yellowing leaves, indicating that *GbCLH* should be involved only in homeostasis of Chl in *G. biloba.*

Materials and Methods

cDNA cloning of chlorophyllase homologue

Amino acid sequences of Chlase from *Arabidopsis thaliana* (*AtCLH1*, AAC13947, and *AtCLH2*, AAF27046), *Chenopodium album* (*CaCLH*, AFF27045), and *Citrus sinensis* cv. Valencia (*Chlase1*, AAF59834) were used to design degenerated primers for reverse transcription-polymerase chain reaction (RT-PCR) cloning of a homologous *G. biloba* gene. The following degenerated primers were used (Y = C or T; M = A or C; S = G or C; H = A, C, or T; V = A, C, or G; N = any base; I = inosine): 5′ CAT GGH TTC ATC STY GTH GCT TC 3′ (forward primer), 5′ GGI CAY TCI CGI GGN GGN MA 3′ (forward primer), 5′ CTT VAG AAA HGC HAC CAC AAT 3′ (reverse primer). Fresh leaves of *G. biloba* were collected in the campus of Osaka University. Total RNA was isolated from the fresh leaves of *G. biloba* using the RNAqueous™-Midi Kit (Ambion, Inc., Austin, TX, USA) with Plant RNA Isolation Aid (Ambion, Inc.) according to the manufacture's instructions. The total RNA was incubated with DNase and then reverse-transcribed with AMV Reverse Transcriptase (Life Sciences, Inc., Petersburg, FL, USA) with Oligo $(dT)_{12-18}$ Primer (Invitrogen Co., Carlsbad, CA, USA). All PCR reactions were cycled 35 times for 30 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. Amplified cDNA was subcloned into plasmid pCR®2.1 (Invitrogen Co.) and sequenced with ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Foster City, CA, USA) using ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The full sequence of Chlase homologue of *G. biloba* was determined using 3′-Full RACE Core Set and 5′-Full RACE Core Set (TaKaRa Bio, Inc., Shiga, Japan). Homology searches were performed with BLAST (Altschul *et al.*, 1990). Sequences were aligned and formated using CLUSTAL W (Thompson *et al.*, 1994) and Boxshade (http://www.ch.embnet.-org/software/BOX—form.html), respectively. Besides the Chlases used for design of degenerate primers, Chlase1, 2, 3 (*Brassica oleracea*) (AAN51933, AAN51934, AAN51935) and Chlase (*Citrus unshiu*) (BAB47176) were also included in the phylogenetic analysis of Chlases.

Protein expression in Escherichia coli

The chlase homologue of *G. biloba* was overexpressed in *E. coli* using a pET expression system (Novagen, Inc., Madison, WI, USA). Chlase expression plasmid was constructed by PCR. The following primers were used to amplify cDNA of the chlase homologue of *G. biloba*: 5′ AAG ATG AAC TCA CTA CTT GCA CAC AG 3′ (forward primer) and 5′ CAA ATA CAT TCC TTT AAT GCA TAA TAC CAT 3′ (reverse primer). The PCR product was amplified again using the following primers to include an extra *Nde*I restriction site at the 5′ end and a *Xho*I site at the 3′ end: 5′ GGA ATT CCA TAT GGT TTT AGT GAA GG 3′ (forward primer) and 5′ CCG CTC GAG TCT GCA AAC TGC CCG 3′ (reverse primer). The PCR product was sequenced and digested by *Nde*I and *Xho*I restriction enzymes. Digested fragments were subcloned into the T7 polymerase expression vector pET-22b(+). The *E. coli* strain BL21(DE3) was transformed with the plasmid and used for expression. Control *E. coli* was transformed with pET-22b(+) vector without insert. Transformants were inoculated in LB medium containing 50 mg/ml ampicillin and cultured until OD_{600} reached 0.6. Recombinant Chlase was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Subsequent protein expression was allowed to proceed for 16 h at 20 °C before harvesting the cells by centrifugation at 10,000 × *g* for 10 min at 4 °C. The recombinant protein was extracted using

BugBuster™ HT Protein Extraction Reagent (Novagen, Inc.) according to manufacturer's instructions.

Enzyme assay

The enzyme solution (0.2 ml) was mixed with the reaction buffer [25 mM (*N*-morpholino)propanesulfonic acid (MOPS) NaOH, pH 7.0, containing 50 mM Chl *a* and 50 mM lauryldimethylamine *N*-oxide, 0.8 ml) and incubated at 30 °C for 30 min. The reaction was stopped by adding 3 ml of acetone/*n*-hexane 1:2 (v/v) and 0.1 ml of 2 M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) HCl, pH 9.0. After vigorously shaking the mixture, the concentration of Chlide *a* in the aqueous layer was spectrophotometrically determined using an absorption coefficient of 76.79 $mM^{-1}cm^{-1}$ at 667 nm (Porra *et al.*, 1989).

The protein concentration was determined by the Bradford method with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard.

Southern hybridization analysis

Genomic DNA was prepared from fresh green leaves of *G. biloba* using DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany). Genomic DNA (30 μg) was digested with restriction enzymes, separated on 1% agarose gel and transferred onto nylon membranes (Hybond-N+; Amersham Biosciences AB, Uppsala, Sweden). ECL Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences AB) was used for the labelling of cloned cDNA of the chlase homologue from *G. biloba* as a probe and signal detection.

Northern hybridization analysis

Total RNA was isolated from fresh green, yellow-green, and yellow leaves as described above. Equal amounts of RNA (10 μg total RNA) was separated on 1% agarose gel containing 2.2 M formaldehyde and blotted onto nylon membranes (Hybond-N+; Amersham Biosciences AB). The cloned cDNA of the chlase homologue of *G. biloba* was used as a probe. Hybridization and washing were carried out with AlkPhos Direct (Amersham Biosciences AB) following the manufacturer's instructions. CDP-Star (Amersham Biosciences AB) was used for signal detection.

Results and Discussion

Isolation of the cDNA encoding chlorophyllase homologue from G. biloba

The cDNA that encodes Chlase was isolated by nested RT-PCR using the degenerated primers. The cDNA sequence, named *GbCLH* (AY292526), and its deduced amino acid sequence is shown in Fig. 1. The *GbCLH* nucleotide sequence was 1,157 bp long and contained a 1,029 bp open-reading frame. The encoded protein consisted of 342 amino acids, and its molecular mass was calculated to be 37,109 Da. The esterase/lipase/thioesterase motif (Ser active site), IPR000379, was found by an InterProScan (http://www.ebi.ac.uk/interpro/scan.html) program (dashed underline in Fig. 1). The sequence "GHSRGG" included the deduced Ser active site (indicated by the star in Fig. 1) (Jacob-Wilk *et al.*, 1999; Tsuchiya *et al.*, 1999) , which is well conserved in known Chlases (underline in Fig. 1).

Expression of recombinant GbCLH in E. coli

Recombinant *GbCLH* was expressed as a His-tag labeled protein in *E. coli.* High Chlase activity was found in the extract of *GbCLH*-expressing *E. coli*, whereas no Chlase activity was seen in the extract of the control *E. coli* (data not shown). Molecular weight of the recombinant *GbCLH* was determined to be about 38 kDa (with His-tag) by both SDS/PAGE and gel-filtration chromatography (data not shown).

Genomic analysis of GbCLH

Southern analysis showed the presence of a single copy of *GbCLH* on the *G. biloba* genome (Fig. 2). The homology with known Chlases is not high (ca. 30–50%), suggesting a possibility that other Chlases are coded in the *G. biloba* genome. A fragment of about 1.6 kbp was found to be labeled after *Eco*RI digestion. This finding led to PCR analysis using the genomic DNA as a template to determine the presence of introns. The amplified products were the same in size as the amplicon with cDNA as a template, which indicated that no intron is in the *GbCLH* gene while an intron exists both in *AtCLH1* and in *AtCLH2.* As *G. biloba* is an early diverging gymnosperm, the intron should have been inserted during the evolution from gymnosperm to angiosperm. Sequence analysis of Chlases from a wide range of

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TTGAAAAACAAAAACGAAGAAGATGAACTCAGTACTTGCACACAGCCATCGGCCATGGTT    60
                                                        M  V

TTAGTGAAGGATGTGTTCAGCGAAGGTCCTTTACCTGTTCAAATCCTCGCAATTCCACAA   120
L  V  K  D  V  F  S  E  G  P  L  P  V  Q  I  L  A  I  P  Q

GCCAACTCATCTCCATGCTCAAAATTAGCAGACAAAAACGGAACTGCAACCACGCCTTCT   180
A  N  S  S  P  C  S  K  L  A  D  K  N  G  T  A  T  T  P  S

CCTTGTCGGCCTCCTAAACCCCTGCTGATCGCTCTTCCTTCCCAACATGGAGATTATCCT   240
P  C  R  P  P  K  P  L  L  I  A  L  P  S  Q  H  G  D  Y  P
                                   ----------------------

CTCATCCTCTTTTTCCACGGCTATGTACTCCTCAATTCCTTCTATTCTCAACTCTTGCGC   300
L  I  L  F  F  H  G  Y  V  L  L  N  S  F  Y  S  Q  L  L  R
----------------------------------------------------------

CATGTTGCTTCCCATGGATACATCGCCATAGCTCCTCAGATGTACAGTGTAATTGGCCCA   360
H  V  A  S  H  G  Y  I  A  I  A  P  Q  M  Y  S  V  I  G  P
----------------------------------------------------------

AATACGACTCCAGAAATAGCCGATGCAGCGGCCATTACAGACTGGTTACGAGATGGACTC   420
N  T  T  P  E  I  A  D  A  A  A  I  T  D  W  L  R  D  G  L
----------------------------------------------------------

TCGGATAATCTTCCGCAAGCTTTAAACAATCATGTGAGGCCCAATTTTGAGAAATTTGTG   480
S  D  N  L  P  Q  A  L  N  N  H  V  R  P  N  F  E  K  F  V
----------------------------------------------------------

          *
CTAGCGGGGCACTCGCGCGGGGGTAAAGTGGCATTTGCACTTGCCCTAGGTCGAGTCTCG   540
L  A  G  H  S  R  G  G  K  V  A  F  A  L  A  L  G  R  V  S
------=========================================-------------

CAGCCATCTTTAAAGTACTCGGCCCTTGTAGGTCTTGATCCAGTCGATGGAATGGGAAAA   600
Q  P  S  L  K  Y  S  A  L  V  G  L  D  P  V  D  G  M  G  K

GATCAACAAACCAGTCATCCTATTCTGTCATACAGAGAGCATTCCTTTGATTTGGGTATG   660
D  Q  Q  T  S  H  P  I  L  S  Y  R  E  H  S  F  D  L  G  M

CCAACATTAGTGGTAGGTTCGGGCCTGGGTCCGTGCAAAAGAAACCCTCTCTTCCCTCCC   720
P  T  L  V  V  G  S  G  L  G  P  C  K  R  N  P  L  F  P  P

TGTGCTCCCCAAGGTGTTAACCACCATGATTTCTTCTACGAATGTGTCGCTCCTGCCTAT   780
C  A  P  Q  G  V  N  H  H  D  F  F  Y  E  C  V  A  P  A  Y

CATTTTGTTGCCTCTGATTATGGGCATCTTGATTTCTTAGACGACGACACCAAAGGAATA   840
H  F  V  A  S  D  Y  G  H  L  D  F  L  D  D  D  T  K  G  I

AGAGGAAAGGCTACTTATTGCCTCTGTAAGAATGGGGAAGCAAGAGAGCCAATGCGGAAG   900
R  G  K  A  T  Y  C  L  C  K  N  G  E  A  R  E  P  M  R  K

TTTAGCGGTGGAATTGTGGTTGCATTTCTTCAAGCATTTCTTGGTGATAATCGTGGAGCC   960
F  S  G  G  I  V  V  A  F  L  Q  A  F  L  G  D  N  R  G  A

CTGAATGATATTATGGTTTATCCTTCACATGCTCCAGTCAAGATTGAGCCTCCAGAGTCT  1020
L  N  D  I  M  V  Y  P  S  H  A  P  V  K  I  E  P  P  E  S

TTGGTTACAGAAGATGTAAAATCCCCAGAAGTCGAATTATTACGCCGGGCAGTTTGCAGA  1080
L  V  T  E  D  V  K  S  P  E  V  E  L  L  R  R  A  V  C  R

TGATGTACCATGGTATTATGCATTAAAGGAATGTATTTGTTATTAAAAAAATATTAAGAA  1140
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GTAAAAAAAAAAAAAAA  1157
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Fig. 1. Nucleotide and deduced amino acid sequences of *GbCLH* (AY292526). The esterase/lipase/thioesterase motif (OPR000379/InterProScan) is represented with a dashed underline. The conserved GHSRGG sequence is represented with a solid line. The serine of the active site is marked with a star.

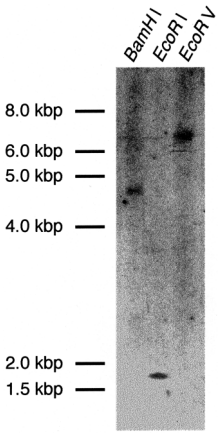

Fig. 2. Genomic Southern analysis of *GbCLH*. Genomic DNA was digested by the indicated restriction enzymes and hybridized with the probe.

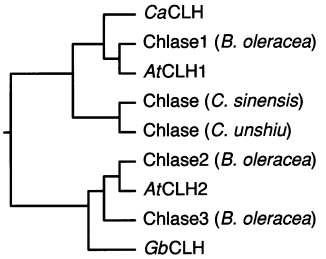

Fig. 4. Phylogeneric relationships of Chlases. The tree was constructed by phylogeneric analysis using CLUSTAL W (Thompson *et al.*, 1994). Accession numbers are: *AtCLH1*, AAC13947; *AtCLH2*, AAF27046; *CaCLH*, AFF27045; Chlase (*C. sinensis*), AAF59834; *GbCLH*, AY292526; Chlase1, 2, 3 (*Brassica oleracea*), AAN51933, AAN51934, AAN51935, respectively; Chlase (*Citrus unshiu*), BAB47176.

gymnosperms should provide new information about the relationship of Chlases between gymnosperms and angiosperms.

Gene expression of GbCLH during autumnal yellowing

In order to investigate the role of *GbCLH* in the autumnal leaf yellowing of *G. biloba*, the gene expression profile of *GbCLH* was examined. The *GbCLH* transcript was observed at 1.2 kb (Fig. 3). The expression level of *GbCLH* decreased significantly with the yellowing of the leaves. This change has been highly correlated to the reduction of Chlase activity and Chl-content (Tang *et al.*, 2000). These results suggest that *GbCLH* is responsible for the homeostasis of Chls but not for the autumnal coloration. In *Arabidopsis*, the expression level of *AtCLH2* was constitutive and did not respond to MeJA (Tsuchiya *et al.*, 1999). The phylogenetic tree predicted that the Chlases are classified into two groups (Fig. 4). It is interesting that the constitutive *AtCLH2* and *GbCLH* belong to the same group, and phytohormone-responsive *AtCLH1* and Chlase (C. *sinensis*) are in the other group (Jacob-Wilk *et al.*, 1999). This information leads us to the hypothesis that the former group should be involved in the homeostasis of Chls while the latter group responds to hormonal stimulation.

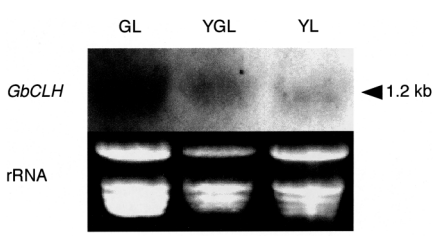

Fig. 3. Seasonal change of *GbCLH* mRNA expression level in the leaves of *G. biloba*. Total RNA was extracted from green (GL), yellow-green (YGL) and yellow (YL) leaves and analyzed by Northern hybridization.

Together with the evidence of our previous study, we can state that the expression level and the activity of Chlase decrease significantly, while Mg-dechelatase increase with the progress of the leaf-yellowing in *G. biloba* (Tang *et al.*, 2000). During this process, the Chl-synthesis of the leaves seems to be suppressed and the Mg^{2+}-flow from the detaching leaves to the surviving trunks is accelerated. In the autumnal coloration of *G. biloba*, the recovery of Mg^{2+} seems to occur prior to the hydrolysis of the phytyl ester. It will be interesting to further examine the relationship between the role and the evolution of this enzyme.

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