

Genes and Biochemical Characterization of Three Novel Chlorophyllase Isozymes from *Brassica oleracea*[†]

Guan-Chiun Lee,[‡] Hanna Chepyshko,[§] Hsiu-Hui Chen,^{\parallel} Chih-Chieh Chu,^{\parallel} Yi-Fan Chou,^{\parallel} Casimir C. Akoh,^{\perp} and Jei-Fu Shaw^{*,§, \parallel}

[‡]Department of Life Science, National Taiwan Normal University, Taipei, Taiwan, [§]Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan, ^{II}Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan, and ^LDepartment of Food Science and Technology, University of Georgia, Athens, Georgia 30602-7610

Three full length cDNAs (BoCLH1, 1140 bp; BoCLH2, 1104 bp; BoCLH3, 884 bp) encoding putative chlorophyllases were cloned from the cDNA pools of broccoli (Brassica oleracea) florets and characterized. The amino acid sequence analysis indicated that these three BoCLHs contained a highly conserved lipase motif (GXSXG). However, only BoCLH3 lacked the His residue which is the component of the catalytic triad (Ser-His-Asp). N-terminal sequences of BoCLH1 and BoCLH2 were predicted to have typical signal sequences for the chloroplast, whereas the plasma membranetargeting sequence was identified in BoCLH3. The predicted molecular masses of BoCLH1, 2, and 3 were 34.7, 35.3, and 23.5 kDa, respectively. The recombinant BoCLHs were successfully expressed in Escherichia coli for the biochemical characterization. The recombinant BoCLH3 showed very low chlorophyllase activity possibly due to its incomplete catalytic triad. BoCLH1 and BoCLH2 showed significant differences in biochemical properties such as pH stability and temperature optimum. Kinetic analysis revealed that BoCLH1 preferably hydrolyzed Mg-free chlorophyll, while BoCLH2 hydrolyzed both chlorophyll and Mg-free chlorophyll at a similar level. Different characteristics between BoCLH1 and BoCLH2 implied that they may have different physiological functions in broccoli. The catalytic triad of recombinant BoCLH2 was identified as Ser141, His247, and Asp170 by site-directed mutagenesis. It suggested that the three broccoli chlorophyllase isozymes were serine hydrolases.

KEYWORDS: Chlorophyllase; isozyme; Broccoli (Brassica oleracea)

INTRODUCTION

Chlorophyllase (chlorophyll chlorophyllidohydrolase, Chlase, EC 3.1.1.4), which catalyzes the hydrolysis of chlorophyll (Chl) to chlorophyllide (Chlide) and phytol (dephytylation), was proposed to be the first enzyme in Chl breakdown during the degreening processes of plants such as leaf senescence, pathogen infection, and fruit ripening (I-4). Although the enzyme has long been known to exist in diverse species, including diatoms (5), chlorella (6, 7), and some angiosperms (6), only a few Chlase genes were isolated. Tsuchiya et al. cloned the first Chlase gene (CaCLH) from the mature leaves of *Chenopodium album* and then isolated two homologues of Chlase genes (*AtCLH1* and *AtCLH2*) in *Arabidopsis thaliana* leaves (8). One *Citrus sinensis* Chlase cDNA (*CsCLH*) was obtained from ethylene-treated citrus fruit peel (9). The *Triticum aestivum* (wheat) Chlase cDNA (*TaCLH*) was identified among expressed sequence tags generated

*Corresponding author. Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan, 40227. Tel: +886-4-22840201. Fax: +886-4-22853813. E-mail: presid@ dragon.nchu.edu.tw. from wheat leaves of etiolated seedlings (10). The recombinant Chlase from *Ginkgo biloba* (GbCLH) was recently characterized, and its subcellular localization was analyzed by transient expression (11).

The deduced amino acid sequences of all cloned Chlase genes share a GXSXG lipase motif containing an active site residue of serine. *AtCLH2* and *CsCLH* encode proteins of 318 and 329 amino acids, respectively, and both contain a predicted N-terminal signal peptide for the chloroplast. *AtCLH1* encodes a protein of 324 amino acids, which has no typical signal sequence. In contrast, *CaCLH* encodes a 347 amino acid protein that contains a typical signal peptide for the endoplasmic reticulum (ER) and a (NPIR) vacuolar-sorting determinant, suggesting that CaCLH is a vacuolar enzyme produced via ER transport. It is still not clear whether various plants or tissues evolve different isoenzymes which are targeted to various organelles for regulating different types of Chl degradation.

Broccoli (*Brassica oleracea*) is an important vegetable crop. The florets senesce and turn yellow rapidly after harvest. Chlase is presumably involved in the breakdown of the Chl. The cloning and characterization of the Chlase gene is essential to understanding the physiological role of Chlase and preventing Chl

[†]GenBank accession numbers: *BoCLH1*, AF337544; *BoCLH2*, AF337545; *BoCLH3*, AF337546.

degradation by transgenic antisense Chlase genes. We have reported three broccoli Chlase genes *BoCLH1*, *BoCLH2*, and *BoCLH3* (GenBank accession numbers AF337544, AF337545, and AF337546). Expression of the antisense constructs of gene *BoCLH1* in broccoli plants using *Agrobacterium tumefaciens*mediated transformation resulted in a phenotype with slower postharvest yellowing during storage (*12*). In the present article, we describe the cloning, expression, and characterization of these three Chlase cDNAs from mature broccoli florets. The primary structures of three deduced proteins were analyzed and compared with other plant Chlases.

MATERIALS AND METHODS

Plant Material. Plants of broccoli (*Brassica oleraceae*, cv. Green king) were grown from seeds in a growth chamber. Tissue samples were harvested 2 weeks after the first cluster of florets appeared. The tissue samples of florets were collected separately. The collected tissue samples were frozen in liquid nitrogen and stored at -70 °C for further use.

Cloning of Broccoli Chlase cDNAs. The total RNA isolated from a freshly harvested broccoli floret was used for cDNA synthesis by a CapFinder cDNA synthesis system (Clonetech, Palo Alto, CA, USA). The cDNA pool and a pair of degenerate primers derived from conserved sequences of reported Chlases were used for PCR amplification. The sequence of the degenerate primers are as follows: CLHdN (5'-GGY-CAYAGYMGMGGWGGVAAAWC-3') and CLHdC (5'-ARCATR-TCCAWRTGHCCRTAMT-3'). A cDNA fragment of about 336 bp in length was amplified and subcloned into a pGEMT-EASY vector (Promega, Madison, WI) for DNA sequence analysis. The sequence of the cloned cDNA fragment that shared high homology to the known Chlase genes was used to design nested primers for cloning 5' and 3' ends of the full length cDNA by the 5'/3' RACE technique. Specific primers corresponding to each terminal sequence was designed for further cloning of various full length cDNA through PCR. The primers derived from the 5' and 3' untranslated regions of each Chlase cDNA were used to amplify the full length cDNAs from the cDNA pool. These primers are Bo-CLHaN (5'-ATACAAATGGCGGGGAAGGA-3') and Bo-CLHaC (5'-GAGT-TTGGAGTCTTTCTGGGGATAC-3') for generating BoCLH1; Bo-CLHbN (5'-AGAGAAAAAAGTGAAAAAAAAAAAC-3') and Bo-CLHbC (5'- GTCAAGATGTCACTTGAGTAAAGAC-3') for generating BoCLH2; and Bo-CLHcN (5'-ACACAAAAAAATATATAACACAAAG-3') and Bo-CLHcC (5'-CACAAGAGGTTTCTTACCTCATA-3') for generating BoCLH3. In order to bypass the misincorporated bases associated with PCR, both strands of the cDNA sequence were confirmed by a direct sequencing strategy using automated DNA sequencers (DSQ-1, Shimadzu; 373A, Applied Biosystems) with commercially available kits (Perkin-Elmer; Amersham Pharmacia Biotech; Wakunaga; Takara Shuzo). The DNA and deduced amino acid sequences were analyzed by the program of SeqWeb version 2 of the Genetics Computer Group (Accelrys Inc.) software package.

Expression Plasmid Construction. The specific forward primers for amplifying the open reading frames (ORFs) of *BoCLH1*, *BoCLH2*, and *BoCLH3* were designed to create an *Eco*RI restriction site upstream from the translation start site. The specific reverse primers were designed to contain a *Xba*I site downstream from the end of the open reading frame of *BoCLH1* and the *Hin*dIII site for both *BoCLH2* and *BoCLH3*. The PCR conditions were as follows: 95 °C for 3 min; 30 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min; and 72 °C for 5 min. PCR products of the amplified three Chlase ORFs were purified by agarose gel electrophoresis and then subcloned in the *E. coli* expression vectors pMAL-c2x (New England BioLabs Inc., Beverly, MA) following the manufacturer's instructions. The recombinant vectors were transformed into *E. coli* Rosseta-Gami B (DE3). Transformants were used to produce recombinant Chlases.

Protein Expression and Purification. *E. coli* transformant with the pMAL-c2x-BoCLH plasmid expressed recombinant Chlases with an N-terminal fusion of maltose-binding protein (MBP). The protein expression and purification were performed according to the procedure recommended by the manufacturer (New England BioLabs, Inc.). The expression of each MBP-fusion Chlase was induced by adding 0.1 mM

Table 1. Oligonucleotide Primers Used for Site-Directed Mutagenesis of BoCLH2

mutagenic primer	nucleotide sequence $(5' \rightarrow 3')^a$			
	reversed primers			
CLH2-H66A	G GTA ACC AGC GAG GAG CAT C			
CLH2-S141A	TTT GCC GCC GCG GGC ATG GCC GG			
CLH2-D170A	ATC CAC CGG AGC TAC ACC GA			
	forward primers			
CLH2-H226A	GGA GTG AAC GCC CGA GAG TT			
CLH2-H239A	T CCA GCG TG <mark>G GCT TTT GTC GCA</mark>			
CLH2-H247A	AG GAT TAC GGA GCT TTG GAC ATG			

^a Underlining denotes the mismatched nucleotide, and bold facing denotes the mutant codon.

isopropyl- β -D-thiogalactopyranoside into the refreshed culture at 37 °C for 6 h. The cells were then harvested and lysed by sonication in TE buffer containing 20 mM Tris-HCl (pH 7.4) and 2 mM EDTA. After high-speed centrifugation (10,000g for 10 min at 4 °C), the resultant supernatant was used as the enzyme source.

MBP-fusion Chlases were purified using an amylose resin affinity column. Native forms of Chlases were prepared from MBP-BoCLH recombinant proteins by cleavage with protease under the following conditions. Purified fusion protein from amylose resin was directly cleaved with Factor Xa protease (0.5 U/10 μ g of fusion protein) (Novagen, Madison, WI) in the column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 1 mM EDTA) with 10 mM maltose for 2.5-3.5 h at room temperature. After protease cleavage, Chlases were further purified by ion exchange column chromatography using a DEAE Sepharose Fast Flow column (Amersham, Pharmacia Biotech, Buckinghamshire, UK) equilibrated with TE buffer (20 mM Tris-HCl, pH 7.4, and 1 mM EDTA). After loading the cleavage reaction mixture onto the column, pure Chlases were eluted with TE buffer containing 200 mM NaCl. Protein concentration was determined with the Bradford dye-binding assay (BioRad, Hercules, CA, USA) using bovine serum albumin (Pierce Biotechnology, Rockford, IL) as a standard.

Chlase Assay. The standard reaction mixture for the activity assay of Chlase contains 10 μ L of enzyme sample, 65 μ L of reaction buffer (100 mM sodium phosphate, pH 7.4, and 0.24% Triton X-100), and 7.5 µL of acetonedissolved Chl a (at a final concentration of 500 µM) (Sigma, St. Louis, MO, USA). The reaction mixture was incubated in a shaking water bath at 40 °C. The amount of product formed had a linear relationship with reaction time within 30 min. Therefore, in the following assay we carried out the reaction for 30 min to measure the initial velocity. Enzyme reaction was stopped by transferring the reaction mixture to a centrifuge tube containing 1 mL of the mixture of acetone/hexane/10 mM KOH = 4:6:1 (v/v). The mixture was vigorously vortexed and centrifuged at 12000g for 2 min for phase separation. The Chlide *a* formed remained in the aqueous acetone layer, while the unreacted Chl a was extracted into the n-hexane layer (13). The absorbance of the aqueous acetone phase was measured at 667 nm for Chlide a with a spectrophotometer. The amount of each product in the acetone layer was estimated from the millimolar extinction coefficient of $81.0 \text{ mM}^{-1} \text{ cm}^{-1}$ for Chlide a (14). One unit (U) of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 µmol of Chl a per min at 40 °C.

Enzyme Characterization. The pH effect on the activity of the purified recombinant Chlase was measured between pH 3.0 to 10.0, using Good's buffer (50 mM Bicine, 50 mM CHPS, sodium acetate, and 50 mM bis-Tris propane). For this purpose, $10 \,\mu$ L of enzyme mixed with 65 μ L of Good's buffer with different pH values and 7.5 μ L of Chl *a* (500 μ M) was incubated at 40 °C for 5 min. The pH stability assay was performed with the enzyme which was incubated with Good's Buffer containing 0.2% Triton X-100 at different pH values from 3.0 to 10.0 for 10 min at 40 °C. Chlase activity was subsequently measured as described above.

The temperature dependence of chlase activity was measured between 20 and 80 $^{\circ}$ C using the standard Chlase assay. Thermal stability profiles were performed with enzymes, which were incubated at different temperatures ranging from 20 to 80 $^{\circ}$ C for 10 min and then chilled on ice. Chlase activity was subsequently measured as described above.

with different substrates such as Chl a, Chl b (Sigma, St. Louis, MO, USA), and pheophorbide (Pheide) a methyl ester (an Mg-free chlorophyll analogue) (Wako Pure Chemical Industries, Japan). The assay reaction contained 10 μ L of enzyme, 65 μ L phosphate buffer, and 7.5 μ L acetone dissolved substrate with concentrations ranging from 50 to 500 μ M. All reactions were carried out at 40 °C for 5 min in triplicate and average values reported. The absorbance of the aqueous acetone phase was measured at 651 nm for Chlide b and 405 nm for Pheide a with a spectrophotometer. The amount of each product in the acetone layer was estimated from the millimolar extinction coefficient of $47.6 \text{ mM}^{-1} \text{ cm}^{-1}$ for Chlide *b* and 77.3 mM⁻¹ cm⁻¹ for Pheide *a* (14). Enzyme kinetic parameters (V_{max} , K_m , and k_{cat}) were determined for

Substrate specificity of the purified recombinant Chlase was determined

three specific substrates. To determine the kinetic parameters, initial

velocities were measured for at least five concentrations of each specific substrate. Product formation had a linear correlation with time under the assay conditions. The data were fitted to the Michaelis-Menten equation, and kinetic parameters were calculated through nonlinear regression using Origin software (version 6.1, UK).

Site-Directed Mutagenesis. The putative catalytic Ser141, Asp170, and four conserved His residues (His66, His226, His239, and His247) of BoCLH2 were altered to alanine by the PCR-based megaprimer method (15). Megaprimer was synthesized by PCR using a forward BoCLH2-NdeI primer (5'-GGAATTCCATATGTCATCTTCTTCATC-3') and a reversed mutagenic primer (Table 1) or using a forward mutagenic primer (Table 1) and a reversed BoCLH2-Xho-HT primer (5'-CCGCTCGAGCT-TTTTAACCTCAAACTC-3'). These mutagenic primers were synthesized by Mission Biotech (Taipei, Taiwan). The resulting PCR products contained

Figure 1. Nucleotide and deduced amino acid sequences of (a) BoCLH1, (b) BoCLH2, and (c) BoCLH3. The sequences of the 5' and 3' untranslated regions
are shown in lower case. The predicted polyadenylation signal sequences are underlined. The conserved lipase motif is shown in dashed underlining, and the
putative active site of the Ser residue is boxed.

(c) $\frac{1}{1}$	acacaaaaaaatatataacacaaagaaatagaagaaggaaaaaATGTCCCCCTCCTTTCT M S P S F L	60 6
61 7	TTCTTTACTTTGTTTTGATAAAGGAAATGTCCTCTTCATCAGCAAACTCCTTTGA F F T L F L I K E M S S S S S A N S F E	120 26
121 27	GGACGCCAAATACAAAACAGATCTTTTAACAGTAGGCTTATCATCTTGCTGCTGGAAAAA D G K Y K T D L L T V G L S S C C W K K	180 46
181 47	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	240 66
241 67	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	300 86
301 87	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	360 106
361 107	ATATAGCATTGCCGGACCAGACCACCATGGATGAGATAAAATCAACGGCAGAGATTATTGA Y S I A G P D T N D E I K S T A E I I D	420 126
421 127	$\begin{array}{cccccccccccccccccccccccccccccccccccc$	480 146
481 147	$\begin{array}{cccccccccccccccccccccccccccccccccccc$	540 166
541 167	$ \begin{array}{cccc} \texttt{ATTTGGATACTCGTCCGACCTAAAGATCTCCGGCATTGATAGGTATAGATGTTGGAACTGT } & \texttt{F} & \texttt{G} & \texttt{Y} & \texttt{S} & \texttt{D} & \texttt{L} & \texttt{K} & \texttt{I} & \texttt{S} & \texttt{L} & \texttt{I} & \texttt{G} & \texttt{I} & \texttt{D} & \texttt{V} & \texttt{G} & \texttt{T} & \texttt{V} \\ \end{array} $	600 186
601 187	$\begin{array}{cccc} TTTTTGGACAAATGGCTATGGCCAATATTCCGGTGAATTTTTCGAGCAATTTGATTGTCG\\ F & U & T & N & G & Y & G & Q & Y & S & G & E & F & F & E & Q & F & D & C & R \end{array}$	660 206
661 207	AMATGACCGGATTGTGGAATCGTAGgattcattgttatgagcactatggtatagtgtaat N D R I V E S \star	720 213
721 781 841	catatatcaaaaacgaagttcgtttgaatgagaaatgaaagtctaaaatagattatttgt aaaatatctatattagaatta gaggtaagaaacctcttgtgtttaaaatggagaagtta taacaaagt <u>tataaa</u> aaactttgtaacaatttggtgtgttagcaaaaaaaaaa	780 840 884

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(a) 1	atacaaATGGCGGGGAAGGAGGACAGTGAGACGTTTTTTCTCGGCGGCGAACTCCTTTGGCG M A G K E D S E T F F S A A T P L A	60 18	(b) ¹ ₁	agagaaaaaaagtgaaaaaaataacaaagagaagaaaaaaATGTCATCTTCTTCATCAA N S S S S R
61	TTTGAGTTAGGCAGCCTTCCAACAACCGTGATCCCCCGCAGACCGGCCAACCGATTG	120	61	GAAACGCCTTTGTGGATGGCAAATACAAACCAGATCTTTTAACAGTGGATTTAGCATCTC
19	F E L G S L P T T V I P A D P S A T D L	38	8	N A F V D G K Y K P D L L T V D L A S R
121	ACCGCACCTCCAAAGCCTGTAATAATCACCTCCCCAACCGTCGCCGGAACTTACCCCGTC	180	121	GCTGTCGCTGCTACAAAACGACGCCATCTTCTTCTCTGACTCCGCCGCCTCCTCCCAAGT
39	T A P P K P V I I T S P T V A G T Y P V	58	28	C R C Y K T T P S S S L T P P P P P K S
181	GTCTTATTCTTCCATGGATTCTATCTTCGTAACTACTTCTACTCTGATGTTATTAACCAC	240	181	CGCTTTTGGTGGCAACGCCGGTGGAGGAAGGAGAATATCCGGTGGTGATGCTCCTCCATG
59	V L F F H G F Y L R N Y F Y S D V I N H	78	48	L L V A T P V E E G E Y P V V M L L H G
241	GTAGCTTCTCATGGCTACATTGTTGTAGCCCCCACAGCTTTGCAGATTTTGCCGCCGGGA	300	241	GTTACCTTCTACAACTCATTTTACTCCCAGCTTATGTTGCATGTCTCTTCCTATGGCT
79	V A S H G Y I V V A P Q L C K I L P P G	98	68	Y L L Y N S F Y S Q L M L H V S S Y G F
301	GGGCAAGTGGAAGTGGACGATGCTGGAAAGTGATGAACTTGGACTTCGAAAAACCTCAAA	360	301	TCATTGTCATCGCTCCGCAGTTATATAACATTGCCGGACCAGACACGATAGACGAGATAA
99	G Q V E V D D A G K V I N U T S K N L K	118	88	I V I A P Q L Y N I A G P D T I D E I K
361	GCTCACCTCCCAAGTTCAGTAAACGCTAATGGCAACTACACCGCACTCGTGGGTCATAGC	420	361	AATCAACGGCGGAGATTATTGATTGGTTATCAGTCGGACTTAACCACTTTCTTCCACCAC
119	A H L P S S V N A N G N Y T A L V <u>G H S</u>	138	108	S T A E I I D W L S V G L N H F L P P Q
421	CGCGGTGGTAAAACCGCGTTTGCGGTTGCGTTAGGCCACGCCGCAACACTAGACCCATCC	480	421	AAGTAACACCCAACCTATCTAAATTCGCACTCACCGGCCATAGCCGCGGCGGCGGCAAAACCG
139	<u>R G</u> G K T A F A V A L G H A A T L D P S	158	128	V T P N L S K F A L T G H S R G G K T A
481	ATCAAGTTTTCAGCTCTTGTAGGAATAGATCCAGTTGCAGGAATCAGCAAATGCATAAGA	540	481	CATTCGCCGTGGCCTTAAAGAAATTTGGATACTCATCGGAACTAAAGATCTCAGCAATTA
159	I K F S A L V G I D P V A G I S K C I R	178	148	F A V A L K K F G Y S S E L K I S A I I
541	ACCGATCCCGAAATCTTAACGTACAAACCGGAATCATTCGACCTGGACATGCCGGTTGCA	600	541	TCGGTGTAGATCCGGTGGATGGGACAGGGAAAGGTAAACAAAC
179	T D P E I L T Y K P E S F D L D M P V A	198	168	
601	GTGATCGGTACGGGTCTCGGACCGAAGAGTAACATGCTGATGCCACCATGCGCACCAGCG	660	601	CCTATGAACCAAACTCATTTAACCTAGAAAAGATGCCTGTTCTAGTTATTGGTTCGGGAC
199	V I G T G L G P K S N M L M P P C A P A	218	188	Y E P N S F N L E K M P V L V I G S G L
661	GAAGTGAACCATGAGGAGTTTTATATTGAGTGTAAGGCTACGAAGGGACATTTCGTGGCT	720	661	TTGGTGAACTTGCCCGGAACCCATTGTTTCCACCGTGTGCACCTACGGGAGTGAACCACC
219	E V N H E E F Y I E C K A T K G H F V A	238	208	G E L Å R N P L F P P C Å P T G V N H R
721	GCGGATTACGGACATATGGATATGTTGGACGATAATTTGCCCGGTTTTGTCGGGTTTATG	780	721	GAGAGTTTTTCCAGGAATGTCAAGGTCCAGCGTGGCATTTTGTCGCAAAGGATTACGGAC
239	A D Y G H M D M L D D N L P G F V G F M	258	228	EFFQECQGPAWHFVAKDYGH
781	GCGGGTTGTATGTGTAAGAACGGTAAAACGAGTGAGATGAGAAGCTTTGTTGGT	840	781	ATTTGGACATGCTTGATGATGATACAAAAGGGCTTAGAGGGAAGAGTTCTTATTGTTTGT
259	A G C M C K N G K R K K S E M R S F V G	278	248	
841	GGAATTGTGGTTGCGTTTCTAAAGTATAGTATATGGGGTGAAATGTCAGAGATTCGACAG	900	841	GTAAGAATGGTGAAGAGAGAAAACCAATGAGGAGATTTATTGGTGGAATTGTTGTGTCGT
279	G I V V A F L K Y S I W G E M S E I R Q	298	268	K N G E E R K P M R R F I G G I V V S F
901	ATTTTGAAGGATCCTTCTGTTTCTCCAGCGAGGCTTGATCCTTCGCCGGAGCTGGAAGAG	960	901	TTTTGATGGCTTATCTGGAAGATGATGATGGTGAAGATCAAAGCTGGGTGTC
299	I L K D P S V S P A R L D P S P E L E E	318	288	L M A Y L E D D D C E L V K I K A G C H
961	GCTTCTGGTTATCTCGTCTAGatttgtggttatgtaacgtattggtatccccagaaagac	1020	961	ATGAAGGTGTTCCTGTTGAAATTCAAGAGTTTGAGGTTAAAAAGTAALttcttttagagc
319	A S G Y L V *	324	308	E G V P V E I O E F E V K K *
1021	tocaaactogt <u>aataaa</u> cttattgcatgtttoactacttt <u>aataaa</u> atattgaatatgta	1080	1021	ttgttttcotatttcaatatacaatagtcacactgottatga <u>aatatt</u> gtttagtctt
1081	tootocaaactatg <u>aataat</u> gtttoceagttacggaaaaaaaaaaaaaaaaaaaaaaaaaaaa	1140	1081	tactcaagtgacatcttgactcataaaaaaaaaa

60 7

120 27

180 47

240 67

300 87

360 107

420 127

 $\frac{480}{147}$

540 167

600 187

660 207

720 227

780 247

840 267

900 287

960 307

1020 321

 $1080 \\ 1104$

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BoCLH2 ORF with single amino acid mutation and were purified with an agarose gel-extraction kit (Quiagen II, Madison, WI). The purified PCR products were digested with *NdeI* and *XhoI* and ligated to an *NdeI/XhoI*-predigested pET-20b vector. The sequences of resulting DNA were confirmed by full-length DNA sequencing. A hexapeptide His₆-Tag was located in frame behind the BoCLH2 and facilitated the purification of recombinant wild-type and mutant BoCLH2s by single-step affinity chromatography.

 Table 2.
 Pairwise Identity of the Amino Acid Sequence between BoCLHs and Other Plant Chlases

	BoCLH1	BoCLH2	BoCLH3	CaCLH	CsCLH	AtCLH1	AtCLH2	TaCLH	GbCLH
BoCLH1 BoCLH2 BoCLH3 CaCLH CsCLH AtCLH1 AtCLH1	100 ^a	38 100	22 47 100	37 31 19 100	42 33 22 35 100	83 38 22 38 41 100	39 85 47 30 34 39 100	43 41 23 36 39 44 40	38 48 25 29 33 38 49
TaCLH GbCLH								100	38 100

^aPercentage of identity was calculated using a ClustalW multiple alignment program with a BLOSUM62 score matrix.

RESULTS

cDNA Cloning of the Broccoli Chlases. Degenerate primers derived from the consensus regions of reported Chlase proteins were used to amplify a partial fragment of BoCLH ORF from the broccoli cDNA pools. Three full-length cDNAs (named BoCLH1, BoCLH2, and BoCLH3) were obtained after 5'/3'RACE. As shown in Figure 1a, the BoCLH1 is an 1140 bp cDNA which contains a 6-bp 5'-untranslated region, followed by a 972 nucleotide open reading frame and a 162-bp 3'-untranslated sequence. Three putative polyadenylation site sequences were observed in the 3'-untranslated region. The open reading frame encodes a protein of 324 amino acids with a predicted molecular mass of 34.7 kDa. The BoCLH2 cDNA is an 1104 bp cDNA which contains a 41-bp 5'-untranslated region, followed by a 963 nucleotide open reading frame and a 100-bp 3'-untranslated sequence (Figure 1b). One putative polyadenylation signal sequence was observed in the 3'-untranslated region. The open reading frame encodes a protein of 321 amino acids with a predicted molecular mass of 35.3 kDa. The BoCLH3 is an 884 bp cDNA which contains a 43-bp 5'-untranslated region, followed by a 639 nucleotide open reading frame and a 202-bp 3'-untranslated sequence (Figure 1c).

BoCLH1	MAGKBOSETFFSAATPLABELGSLPTTVIPADPSATDLTAPPKPV	45
BoCLH2	MSSSSRNAFVDCKYKPDLLTVDLASRCRCYKTTPSSSLTPPSDPKSL	48
BoCLH3	MSPSFLFFTLFLIKEMSSSSANSFEDCKYKTDLLTVGLSSCCWKKPSSSPTPQSPPKR	60
CaCLH	MAKLLLLIFGVFIFVNSQAQTFPTILEKHNSEKITDVEHKCNFQVTNNPIRVKRYEFSAP-EPL	63
CsCLH	MAAMVDAKPAAS-VQGTPLLATATLPV <mark>E</mark> TR <mark>G</mark> IYSTKRITLETSSPSSPP <mark>DPKP</mark> L	53
AtCLH1	MAAIEDSPTFSSVVTPAABEICSLPTTEIPVDPVENDSTAPPKPV	45
AtCLH2	RCCKITPSSRASP-SDPKOL	45
TaCLH	MAAAAPAETMNKSAAG-AEVPEAFTSVEOPCKLAVEAIQVDENAAPATPPIEV	51
GbCLH	MVLVKDVESECPLPVQILAIPQANSSPCSKLADKNGTATTPSPCRDPKPL	50
	•	
BoCLH1	IITSPTVAGTYPVVLFFHGFYLRNYFYSDVINHVASHGYIVVAPQLCKILPPGQQVEVDDAGKVINWTSKNLKAHLP	122
BoCLH2	LVATEVEEGEYPVVMLLHGYLIYNSFYSOLMLHVSSYGFIVIAPOLYNIAGPDTMDELKSTAEIIDWLSVRUNHFLP	125
BoCLH3	LVATPVEEGEYPVVMLLHGYLLYNSFYSOIMLHVSSHGFIVIAPOLYSIAGPDTMDEIKSTAEIIDWLSVGLNHFLP	137
CaCLH	IIISPKEAGVYPVLLFIHGTMLSNEDYSLFFNYIASHGFIVVAPKLFRLFPPKLPSQQDEIDMAASVANMMPLYLQVVLQ	143
CsCLH	IIVTPAGKGTFNVILFLHGTSISNKSYSKIFDHIASHGFIVVAPOLYTSIPPPSATNELNSAAEVAEWLPQGLQQNLP	131
AtCLH1	RITCPTVAGTYPVVLFFHGFYLRNYFYSDVLNHIASHGYILVAPQLCKLLPPGGQVEVDDAGSVINWASENLKAHLP	122
AtCLH2	LVATPVEEGDYPVVMLLHGYLLYNSFYSOLMLHVSSHGFILIAPOLYSIAGPDTMDEIKSTAEIMDWLSVGLNHFLP	122
TaCLH	${\bf LIVAPKDAGTYPVAMLLHGFFLHNHFYEHLLRHVASHGFIIVAPOFSISIIPSGDAEDIAAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAKVADWLPDGLPSVLPACAAAAKVADWLPDGLPSVLPACAAAAKVADWLPDGLPSVLPACAAAAKVADWLPDGLPSVLPACAAAAKVADWLPDGLPSVLPACAAAAKVADWLPDGLPSVLPACAAAAKVADWLPDGLPSVLPACAAAAKVADWLPDGLPSVLPACAAAAKVADWLPDGLPSVLPACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA$	129
GbCLH	LIALPSQHGDYPLILFFHGYVLLNSFYSQLLRHVASHGYTAIAPQMYSVIGPNTTPEIADAAAITDWLRDGLSDNLP	127
	* * * * * *	
BoCLH1	SSVNANGNYTALVGHSRGGKTAFAVALGHAATLDPSIKFSALVGIDSVAGISKCIRTDPEILTYKPESFDLD-MPV	197
BoCLH2	PQVTPNLSKFALTGHSRGGKTAFAVALKKFG-YSSELKISAIIGVDPVDGTGKGKQKQTPPPVLTYEPNSFNLEKMPV	200
BoCLH3	PQVTPNLSKFALSGHSRGGKTAFALALKKFG-YSSDLKISALIGIDVGTVFWTNVFWTN	190
CaCLH	RYV-TGVEGDLEKLAISGHSRGGKSAFALALGFSN-IKLDVTFSALIGVDPVAGRSVDDRTLPHVLTYKPNSFNLS-IPV	220
CsCLH	FONTEANVSLVAVMGHSRGGQTAFALSLRYGFONTGLDPVAGTSKTTGLDPSICSDSFDFS-IPV	196
AtCLH1	TSVNANGKYTSLVGHSRGGKTAFAVALGHAATLDPSITFSALIGIDPVAGTNKYIRTDPHILTYKPESFELD-IPV	197
AtCLH2	AQVTPNLSKFALSGHSRGGKTAFAVALKKFG-YSSNLKISTLIGIDPVDGTGKGKQTPPPVLAYLPNSFDLDKTPI	197
TaCLH	KGVEPELSKLALAGHSRGGHTAFSLALGHAKTQLTFSALIGLDPVAGTGKSSQLQPKILTYEPSSFGMA-MPV	201
GbCLH	QALNNHWRPNFEKFVTA GHSRGGKVAFALAL GRVSQPSLKYSALVGLDPVDCMGKDQQTSHPITSYREHSEDLG-MPT	204
	<u>• *</u>	
BoCLH1	AVIGTGLGP-KSNMLMPPCAPAEVNHEEFYIECK-ATKGHFVAADYGHMDMLDDNLPGFVGFMAG-CMCKNGKRKKSEMR	274
BoCLH2	LVIGSCIGELARNPLFPPCAPTGVNHRBLFQECQGPAW-HFVAKDYGHIDMLDDDTKGLRGKSSY-CICKNG-EERKPMR	277
BoCLH3	GYGQYSGCRNDR	209
CaCLH	TVIGSGLGNHTIS-CAPNH <mark>USHQO</mark> FYDECK-ENSSHFVITK <u>VGHMDMINE</u> FRLSPIAVTMS-IMCAQSFRPKATMR	293
CsCLH	TVIGTGLGGVARCITACAPEGANHEEFFNRCKNSSRAHFVATDYGHMDILDDNPSDVKSWALSKYFCKNCNESRDPMR	274
AtCLH1	AVVCTGLCP-KWNNVMPPCAPTDLNHEEFYKECK-ATKAHFVAADYCHMDMLDDDLPGFVGFMAG-CMCKNCQRKKSEMR	274
AtCLH2	LVIGSGLGETARNPLFDPCAPPGVNHRBFFRBCOGPAW-HFVAKDYGHLDMLDDDTKGIRGKSSY-CLCKNG-EERRPMR	274
TaCLH	LVIGTGLGEEKKNIFFPPCAPKDVNHAEFYRECR-PPCYYFVTKDYGHLDMLDDDAPKFITCVCKDGNGCKGKMR	275
GbCLH	LVVGSGLCPCKRNPLF <u>PPCAP</u> QGVNHHDFFYECVAPAY-HFVASDYGHLDFMDDDTKGIRGKATY-GLCKNG-EAREPMR	281
BoCLH1	SFVGGIVVATLKYSIWGEMSEIRQILKDPSVSPARLDPSPELEEASGYLV	
BOCLH2	RFIGERVESTMAYLEDDDCELVKIKAG-CHEGVPVEIQEFEVKK	
BOCTH3		
CaCLH	KTLEGTIMVATENATERDUGRQYYAITANKSLAYINLFAEKKEFNFG-FATTYAQL 347	
CSCLH	RCVSGI WANTEKDFFYGDAEDFRONLKDPSFANIKLD-SVEYIDASSMLTTTHVKV 329	
ACCLH1	SFVGGTVVATTKISLWGEKABIRLUVKDPSVSPAKLDPSPELEEASGIFV	
AtCLH2	RFVEGLVVISEDKATLEGDDRELVKNKDG-CHEDVPVELQEFEVIM	
TACLH	KCVACTMYATTMAALGEKDADLEATUKDPAVAITTLDPVEHVA	
GDCLH	KFSGCFVVATDFOAFEGDNKGAENDEMVYPSHADVKIEPPESLVIEDVKSPEVELLRRAVCR 342	

Figure 2. Alignment of amino acid sequences of various Chlases from *B. oleracea* (BoCLH), *C. album* (CaCLH), *C. sinensis* (CsCLH), *A. thaliana* (AtCLH), *T. aestivum* (TaCLH), and *G. biloba* (GbCLH). Protein sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index. html). The identical amino acids among at least six out of nine sequences are shaded in black and similar amino acids in gray. The conserved catalytic residues (Ser-His-Asp) are indicated by an asterisk, and the other three conserved His residues are indicated by circle.



Figure 3. SDS—PAGE of the recombinant BoCLH1, BoCLH2, and BoCLH3. (a) Expression and purification of BoCLH-MBPs. Lane 1, purified MBP-BoCLHs with amylose resin; lane 2, soluble fraction of total cell protein; and lane 3, total cell protein. (b) Purification of the recombinant BoCLH2. Lane 1, recombinant BoCLH2-MBP fusion protein purified with amylose resin; lane 2, proteolytic cleavage of purified BoCLH2-MBP with Factor Xa; and lane 3, purified native BoCLH2 enzyme after proteolytic cleavage; lane M, molecular weight standards. SDS—PAGE was performed in a 12% polyacrylamide slab gel. Protein bands were detected by staining with Coomassie Brilliant Blue.

One predicted polyadenylation signal sequence was observed in the 3'-untranslated region. The open reading frame encodes a protein of 213 amino acids with a predicted molecular mass of 23.5 kDa.

Analysis of the Deduced Amino Acid Sequence. As shown in Table 2, the deduced amino acid sequences of three BoCLHs show 22% to 47% of pairwise identity. The broccoli BoCLH1 and BoCLH2 have high homology with Arabidopsis AtCLH1 and AtCLH2, respectively. BoCLH1 shows the highest identity (83%) to the Arabidopsis Chlase AtCLH1, and BoCLH2 shows the highest identity (85%) to AtCHL2. This means that BoCLH1 and AtCHL1 may be orthologues with similar function and that the same relationship might occur between BoCHL2 and AtCLH2. However, BoCLH3 appears to be a unique novel type of Chlase since it is much smaller. In the N-terminal regions, BoCLH3 (residues 1-185) has a higher identity percentage (79%) with BoCLH2 (residues 1-175) than that (35%) with BoCLH1 (residues 1-173); however, it has a shorter C-terminal sequence compared with that of BoCLH2. It is possible that BoCLH2 and BoCLH3 evolved from the same ancestor gene. The N-terminal sequences of BoCLH1 and BoCLH2 were predicted to have typical signal sequences for the chloroplast by CELLO v.2.5, a subcellular localization predictive system (16), whereas BoCLH3 was predicted to be a plasma membrane protein.

The three deduced protein sequence of broccoli Chlases were aligned with those of other reported Chlases using the ClustalW program (17) (Figure 2). The core domain, containing the lipase Ser active site motif GHSRG, is highly conserved among the members of Chlase family. However, the N- and C-terminal sequences are quite variable in length and in composition. Several crystal structures of lipases show conserved spatial arrangement of the catalytic serine in a sharp turn between a β -strand and α -helix, which is called the β -Ser- α motif (18). From the secondary structure prediction using the PREDICTPROTEIN server



Figure 4. Effect of pH on the activity and stability of (a) BoCLH1 and (b) BoCLH2. The activity and the stability are indicated by solid circles and open circles, respectively. Specific activity was measured according to the standard Chlase assay.

(http://www.predictprotein.org/) (19), the three broccoli Chlases showed the conserved β -Ser- α folding pattern. The phylogenetic analysis of esterases and lipases using the peptide fragment sequence revealed that the Chlases are a distinct group of α/β hydrolases with active site serine.

Chlase Protein Expression. Three recombinant broccoli Chlases were expressed in *E. coli* for enzyme activity study. We subcloned the full-length ORFs of Chlase genes under the control of the *tac* promoter in the pMAL-c2x expression vector. The transformants of *E. coli* Rosseta-Gami B (DE3) harboring a recombinant vector expressed recombinant Chlases in a high yield after IPTG induction. We obtained soluble and functional BoCLH1, 2, and 3 as MBP-fusion proteins of molecular sizes 77.4, 78.0, and 66.2 kDa, respectively. After protease cleavage to remove the MBP moiety, we obtained the native BoCLHs (**Figure 3**). We noticed that the BoCLH3 enzyme showed very low Chlase activity; therefore, all further experiments were performed with BoCLH1 and 2.

Biochemical Characterization. Experiments were performed to determine the biochemical properties of the purified recombinant Chlases. The optimal pH was investigated at a pH range between pH 3.0 and 10.0. Figure 4 shows that the pH optima for BoCLH1 and 2 were pH 7.0 and pH 8.0, respectively. BoCLH1 was more acid-active because about 50% of activity was found at pH 4.0, and broad pH stability was found between pH 5.0 and 8.0. On the contrary, BoCLH2 was more basic-active because about 80% of activity was found at pH 9.0, while BoCLH1 showed only 12% of

activity at the same pH value. The pH stability also showed that BoCLH2 was stable between pH 8.0 and 10.0.

The temperature optimum was measured between 20 and 80 °C (Figure 5). BoCLH1 and BoCLH2 showed optimal enzyme activities at 30 and 60 °C, respectively. BoCLH1 showed a 50% decrease in activity at 45 °C, while BoCLH2 showed it at 65 °C. However, these two enzymes showed similar thermostability because after incubation at 60 °C for 10 min, they all lost about 50% of activity.

Kinetic Studies. The kinetic parameters were investigated with various substrates including Chl *a*, Chl *b*, and Pheide *a* methyl ester. The steady-state kinetic parameters such as V_{max} , K_m , and k_{cat} for various substrates were calculated by fitting the data to the Michaelis–Menten equation and summarized in **Table 3**.



Figure 5. Effect of temperature on the activity and stability of (**a**) BoCLH1 and (**b**) BoCLH2. The activity and the stability are indicated by solid circles and open circles, respectively. Specific activity was measured according to the standard Chlase assay.

Kinetic analysis revealed that BoCLH1 hydrolyzes three substrates more rapidly than BoCLH2. Furthermore, BoCLH1 showed distinct substrate specificity for the Pheide *a* methyl ester. The catalytic efficienciy (k_{cat}/K_m) for the Pheide *a* methyl ester is about 20 to 30 times higher than that for the other two substrates due to BoCLH1's high binding affinity for the Pheide *a* methyl ester (i.e., the lowest K_m).

Site-Directed Mutagenesis. Because BoCLHs have a lipase motif (G-X-S-X-G), which is conserved in the alignment of various Chlases (Figure 4), we believed that BoCLHs might have a catalytic triad (Ser-His-Asp/Glu) similar to lipases, and therefore we introduced mutations into the putative catalytic residues of BoCLH2. The recombinant proteins of mutant and wild-type BoCLH2s were purified for the standard Chlase assay. Table 4 shows the specific activities of various recombinant BoCLH2s using Chl a as a substrate. The mutations at the putative catalytic residues (Ser141 and Asp170) caused the loss of Chlase activity as expected. Four mutant BoCLH2s with a single mutation at different conserved His also showed significantly decreased activities. In particular, H247A almost lost activity just as S141A and D170A did. These results showed that three residues (S141, D170, and H247) are important for the Chlase activity in BoCLH2 and that they might be the members of catalytic triad.

DISCUSSION

Chlase plays an essential role in Chl degradation. The isolation and characterization of three Chlase genes from broccoli florets represent an important step toward understanding the molecular mechanism of the Chl breakdown process and the biological function of Chlase isozymes in floret development.

The subcellular localization prediction revealed that BoCLH1 and BoCLH2 might be chloroplast enzymes. However, BoCLH3 did not contain any typical chloroplast signal sequence. In vitro, BoCLH1 and BoCLH2 had significantly higher Chlase activity than BoCLH3. Our present study suggested that in broccoli chlorophyll may be degraded in the chloroplast as previously proposed (20). Chl was found to secrete from senescing chloroplast to cytoplasm (21), which implies that Chl could be also degraded in the cytosol. Tsuchiya et al. speculated that Chl degradation in *C. album* might exist in the vacuole since CaCLH

Table 4. Specific Activity of Wild-Type and Mutant BoCLH2s

mutation	specific activity ^a (U \times mg ⁻¹)
H66A	0.372
S141A	ND
D170A	ND
H226A	0.52
H239A	0.149
H247A	0.074
Wild type	7.43

^a The enzyme activity was determined by measurement of the absorbance of the aqueous acetone phase with Chlide *a* at 667 nm. One unit of Chlase activity was defined as described in Materials and Methods.

Table 3. Steady-State Kinetic Parameters of BoCLH1 and BoCLH2

	substrate	$V_{\rm max}~({\rm mM/min} imes{\rm mg})$	$K_{\rm m}$ (μ M)	$k_{\rm cat}({\rm s}^{-1})$	$k_{\rm cat}/K_{\rm m}~(10^{-5} \times {\rm s}^{-1}~\mu{ m M}^{-1})$
BoCLH1	Chl a	12.40 ± 0.75 ^a	465.92 ± 64.46	0.072 ± 0.005	15.51
	Chl b	11.23 ± 0.51	651.63 ± 113.25	0.072 ± 0.003	11.05
	Pheide a methyl ester	44.80 ± 0.52	76.37 ± 2.72	0.259 ± 0.003	339.67
BoCLH2	Chl a	4.33 ± 0.06	307.93 ± 6.88	0.0024 ± 0.00	0.78
	Chl <i>b</i> Pheide <i>a</i> methyl ester	$\begin{array}{c} 6.57 \pm 0.11 \\ 6.77 \pm 0.21 \end{array}$	$\begin{array}{c} 310.73 \pm 8.75 \\ 143.14 \pm 8.42 \end{array}$	$\begin{array}{c} 0.0037 \pm 0.00 \\ 0.0039 \pm 0.00 \end{array}$	1.19 2.72

^aValues are the means \pm SD from three independent experiments.

contains a typical signal peptide for ER in the N-terminal region (8). Therefore, various plants might evolve different Chl degradation mechanisms and different Chlase regulations. Immunolocalization and Chlase transport experiments are currently underway to confirm our hypothesis.

Previously, Tsuchiya et al. have identified the catalytic triad of CaCLH as Ser162, Asp191, and His262, which is essential for Chlase activity (22). In our present study, introduction of mutations into the putative catalytic triad of BoCLH2 (Ser141, Asp170, and H247) also showed the loss of Chlase activity (Table 4). Sequence alignment reveals that these amino acid residues are conserved with the catalytic triad of CaCLH and also conserved in all other Chlases except BoCLH3, which does not have the catalytic His (Figure 2). This strongly suggested that BoCLHs may be similar to those of serine hydrolases. The absence of the catalytic His residue in BoCLH3 may explain why it had very low Chlase activity. BoCLH3 may have some other substrate specificity or enzyme function other than Chlase, and it might play a different role in Chl degradation from the other two BoCLH isozymes. Further molecular and biochemical investigations are needed to elucidate the actual function of BoCLH3.

Kinetic analysis revealed that BoCLH1 preferably hydrolyzed the Pheide *a* methyl ester which resembles the Mg-free chlorophyll pigment, pheophytin (Phein), and that BoCLH2 hydrolyzed both the Pheide *a* methyl ester and Chl at a similar level. Recently, Schelbert et al. found that pheophytinase (PPH), a chloroplast-located and senescence-induced hydrolase which specifically dephytylates Phein yielding Pheide, is an important component of the chlorophyll breakdown pathway in *Arabidopsis thaliana* (23). We propose that as a whole, BoCLH1 resembling PPH degrades Chl into Pheide via Phein, not Chlide, in broccoli, even though the amino acid sequence similarity is very low between these two enzymes. However, BoCLH2 may degrade Chl into Pheide via both Phein and Chlide. It suggests that different Chlase isozymes may be involved in different Chl breakdown pathways.

Isoforms of enzymes presumably have different biochemical properties and/or regulations. Previously, we have shown that isoforms of *Candida rugosa* lipase have different catalytic efficiencies, substrate specificities, and thermostabilities and show differential expression in the presence of different culture conditions (24, 25). C-terminal deletion of rubisco activase has been shown to have a regulatory effect on both ATP hydrolysis and rubisco activation (26). Therefore, it is logical to assume that the three broccoli Chlase isoforms should have different kinetic properties and regulatory mechanisms. Experiments are currently underway to confirm the actual subcellular localization of BoCLHs and the roles played during the postharvest senescence of broccoli.

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