Characterization of a chitinase gene encoded by virus-sensitive Chlorella strains and expressed during virus infection

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Ali Mohammed M. Ali; Takeru Kawasaki1 and Takashi Yamada*
Department of Molecular Biotechnology, Graduate School of ADSM, HiroshimaUniversity, 1-4-1 Kagamiyama, Higashi-Hiroshima 739-8530,1 Japan
*Corresponding author. Tel: +81-82-424-7752- Fax: +81-82-424-7752 e-mail:tayamad@hiroshima-u.ac.jp

ABSTRACT

A chitinase gene (hChti-1) was found and characterized in a few strains of Chlorella species that are sensitive to Chlorella viruses. A cDNA clone of hChti-1 obtained from Chlorella strain NC64A contained a 401 aa [1206 bp] open reading frame, whose amino acid sequence was most similar to that of family 18 catalytic domain I of dual-structured chitinase vChti-1 encoded by Chlorella virus CVK2. The expression of hChti-1 was induced by virus infection and functional chitinase protein was produced. Since the hChti-1 gene was strictly limited to virus-sensitive strains, its occurrence seems to be involved in the virus infection mechanism. This is the first report of chitinase encoded and produced by unicellular algae.

Key words: *Chlorella chitinase, family 18 glycosyl hydrolase, vChti-1, Chlorella virus.*

INTRODUCTION

Chitinase (EC 3.2.1.14) catalyzes the hydrolysis of chitin, a linear homopolymer of β-1,4-linked N-acetylglucosamine (GlcNAc) residues. The enzymes are produced by a wide range of organisms including bacteria (Clark and Tracey, 1956; Watanabe *et al.* 1990; Flach *et al.*, 1992)), fungi (Gooday *et al.*, 1986), higher plants (Boller, 1985; Collinge *et al.*, 1993), insects (Koga *et al.*, 1983), crustaceans (Lunt and Kent, 1960), and some vertebrates. Bacteria produce chitinases to meet nutritional needs. In most cases, they produce several chitinases to hydrolyze the diversity of chitins occurred in nature. A major role of chitinases produced by fungi, crustaceans, and insects is modification of their structural constituent, chitin, in developmental processes or morphogenesis (Sahai and Manocha, 1993). The proposed role of plant chitinases is a defence mechanism against chitin-containing pathogens or insects (Collinge *et al.*, 1993).

Based on amino acid sequence similarity, chitinases of various organisms are classified on five classes, from class I to class V (Cohen-Kupiec and Chet, 1998). These classes can be grouped into two families of glycosyl hydrolases, family 18 and 19 (Henrissat and Bairoch, 1993; 1996). When the chitinase hydrolyzes a β -1, 4 linkage, the GlcNAc at the reducing end contains an asymmetric carbon 1, which exists as two different stereoisomers, configuration a or b Chitinases of family 19 invert the anomeric configuration of the

hydrolyzed GlcNAc residue. While chitinases of family 18 retain the anomeric configuration (Iseli *et al.*, 1996). Chitinases from Class I, II, and IV are of plant origin and make up the family 19 glycosyl hydrolases (Sahai and Manocha, 1993; Hamel *et al.*, 1997). Class III chitinases are mainly plant and fungal in origin (Hamel *et al.*, 1997) and belong to the family 18 glycosyl hydrolases. Class V is mainly comprised of bacterial chitinases, which are also of the family 18 glycosyl hydrolases. Such a wide variation in the structure and function of chitinases raises interesting questions about evolution of those enzymes.

Recently, a peculiar structural feature of chitinase encoded by a virus of algae, *Chlorella* virus CVK2, has been reported (Hiramatsu *et al.*, 1999): The CVK2 gene (*vChti-1*) codes for an open reading frame of 2,508 bp, corresponding to 836 amino acids (aa). The predicted amino acid sequence contained two sets of a family 18 catalytic domain that is responsible for chitinase activity. Each of the domains showed amino acid sequence homology to different chitinases of the bacterial subfamily-C (Watanabe *et al.*, 1993). The first catalytic domain is most similar to the catalytic sequence of *Saccharopolyspora* (*Streptomyces*) *erythraeus* (30.0% identity), whereas the second domain resembles that of *Ewingelle americana* (34.7% identity). The two catalytic domains are connected by a short linker sequences enriched in proline residues. This structure suggested different origins for the two domains of vChti-1 chitinase (Hiramatsu *et al.*, 1999). A similar composite structure of a chitinase A181/182R has also been reported for *Chlorella* virus PBCV-1 (Sun *et al.*, 1999).

Recently, Hiramatsu *et al.* (Hiramatsu *et al.*, 2000) revealed that the first catalytic domain on a C-terminal-truncated derivative of vChti-1 generated exclusively chitobiose from chitotetraose, chitohexaose, and colloidal high-molecular mass chitin in the enzyme reaction. This property is of a typical exochitinase. Contrasting to this, N-acetylglucosamine was produced from chitobiose as well as chitooligosaccharides by the second catalytic domain on an N-terminal-truncated derivative of vChti-1. Therefore, the second domain possessed N-acetylglucosaminidase activity as well as endochitinase activity. The presence of two catalytic domains with different enzymatic properties in the viral enzyme seems to be necessary for hydrolyzing the natural substrate in a cooperative fashion. The origin and molecular evolution of such a composite chitinase is of great interest.

In this work, we have found and characterized a chitinase gene (*hChti-1*) encoded on the genomic DNA of *Chlorella* strain NC64A , whose ORF showed a significant aa sequence homology with vChti-1 protein. *hChti-1* was specifically expressed and the enzymatically active protein was produced in the cells of *Chlorella* hosts responding to virus infection. To our knowledge, this is the first report on a chitinase encoded by unicellular algae.

Cells and virus

Cells of *Chlorella* strain NC64A (Muscatine *et al.*, 1967) were cultured in a modified Bold's basal medium (MBBM) as described previously (Van Etten *et al.*, 1983). *Chlorella* virus CVK2 was a large plaque-forming virus isolated in Kyoto, Japan

(Yamada *et al.*, 1991). For genomic Southern blot hybridization, *C. vulgaris* C-169 and *C. ellipsoidea* C-87 (211-1a) were obtained from the algal culture collection of the Institute of Molecular and Cellular Biosciences, The University of Tokyo. *C. prototechoides* 211-6 and *Chlorella* sp. SAG-241-80 were from the Algal Culture Collection, Plant Physiology Institute, University of Goettingen. *Paramecium bursaria* zoochlorella strain F36-ZK was provided by Nobutaka Imamura, Ritsumeikan University (Hoshina *et al.*, 2004). Strains C-87 and C-169 were cultured in MBBM at 25 °C in light and strains 211-6 and F36-ZK were cultured in a medium containing NH_4Cl as the sole nitrogen source and thiamine according to Douglas and Huss (Douglas and Huss, 1986). The strain SAG-241-80 was grown in Jaworski's medium (Thompson *et al.*, 1988) supplemented with 0.2% Lab-lemco powder (Oxoid, Hampshire, UK).

Preparation of DNA and RNA

DNA was isolated from cultured *Chlorella* cells and purified virus particles by phenol extraction, as described previously (Yamada *et al.*, 1991). For Southern blot hybridization, DNA restriction fragments separated by agarose gel electrophoreis were transferred to nylon filters (Biodyne, Pall Biosupport, Michigan, USA) and hybridized with probes labeled with fluorescein (Gene Images kit, Amersham Pharmacia Biotech, NJ, USA), and detected with a CDP-Star detection module (Amersham Pharmacia Biotech). The total RNA was isolated from uninfected and virus-infected *Chlorella* cells at various times postinfection (Kawasaki *et al.*, 2004). For Northern blot hybridization, the RNA was separated by 1.5% agarose gel with formamide, blotted onto nylon filters, and hybridized with ^{32}P-labeled probes under standard conditions. Total RNA for real-time PCR was prepared from *Chlorella* cells (strain NC64A) cultivated under various stressed conditions including in complete darkness, treating with high salt concentration (0.5 M NaCl) for 2 hrs, at different temperatures (9, 25, and 40°C) for 2hrs,, at different pH (2, 3, 9, and 10) for 2hrs,, treated with 1% chitinase (chitinase RS, Pias Corp., Osaka, Japan) for 30 min at 25 °C, treated with 2% cellulase (cellulase Onozuka R10, Yakult, Tokyo, Japan) for 30 min at 25 °C, and infection with CVK2 for 10 min at 25 °C. All treatments were carried out in a 200 ml culture containing *Chlorella* cells at a late exponential growth phase (10^7 cells/ml).

Construction of a cDNA library

A cDNA library was constructed using a Time-Saver cDNA synthesis kit (Amersham Pharmacia Biotech) with reverse-transcribed poly(A)$^+$ RNA isolated from CVK2-infected *Chlorella* cells at 20 min postinfection (p.i.). cDNA was ligated to a lambda ZAPII vector and packaged with a Giga-pack Gold packaging kit (Stratagene, La Jolla, CA), according to the manufacturer's instruction. Oligo (dT) primers and *Eco*RI/*Not*I adapters were used in the construction of the library. This cDNA library was screened for specific sequences with radiolabeled probes as described (Kawasaki et al., 2004).

DNA sequencing and sequence analysis

DNA sequences were determined by the standard chain-termination method using an AutoRead sequencing kit and an ALF DNA sequencer (Amersham Pharmacia Biotech). DNA sequences were compiled and analyzed with the DNASIS computer program (Hitachi

Software, Tokyo, Japan). The sequence determined for the hChti-1 gene was deposited to the DDBJ/EMBL/GenBank databases under accession no. AB262188.

Quantitative Real-time PCR

Real-time PCR was performed using a Line Gene Fluorescence quantitative detection system (BioFlux, Tokyo) with total RNA-based cDNAs prepared from *Chlorella* strain NC64A cells cultivated under different stressed conditions. For the quantitative determination of transcripts of *hChti-1*, a PCR mixture containing SYBR-green (SYBR premix *ExTaq*, Takara Shuzo, Kyoto, Japan) was used. PCR reactions in a final volume of 10 µl, containing 5 µl PCR mixture, 1 µl diluted cDNA and 0.5 µM of each oligonucleotide primer were carried out under the following conditions: 1 min at 95 °C, 45 cycles (at 94 °C for 15 sec;55 °C for 15 sec; 72 °C for 30 sec). The following primers were used: forward, 5'-GCT GAA CGA TGT GGA GTC TG -3'; reverse, 5'-ATG GAG GAG GAG TGG ATG -3' (DDBJ accession No.AB262188); forward *alpha-tubulin*, 5'-AGG AGG TGG ACG AGA T-3'; reverse *alpha-tubulin*, 5'-AGG GAT GTC GCA GAT GGA-3' (M. V. Graves, personal communication). At the end of the program, the specificity of the primer set was confirmed by a melting curve analysis (65-95 °C with a heating rate of 0.5 °C/min). The mRNA of alpha-tubulin was used to normalize the expression ratio of *hChti-1* in cDNAs obtained from *Chlorella* cells incubated under different conditions. To obtain more reliable results, all reactions were performed in triplicate.

Preparation of *Chlorella* protein and Western blot analysis

Total protein was prepared from a liter culture of *Chlorella* strain NC64A grown in MBBM containing 0.01% ethyleneglycol chitin (Seikagaku Corp, Tokyo Japan). Cells at a late exponential growth phase (10^7 cells/ml) were harvested by centrifugation at 1,000 g for 10 min at 4°C and homogenized in a mortar with a pestle under liquid nitrogen. The homogenate was suspended in the extraction buffer containing 25 mM Tris-HCl (pH 7.5) containing 0.14 M NaCl, 5 mM EDTA, a proteinase mixture EASYPack complete kit (Roche Applied Science, NY, USA) (1 mM PMSF, 5 µg/ml of leupeptin, and 1 µg of pepstatin), mixed by vortexing for 3 min at 4 °C, and centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was recovered and the protein concentration was determined using a dye-binding assay kit (Bio-Rad, CA, USA) with bovine serum albumin as the standard. Proteins were electrophoresed on an SDS-10% polyacrylamide gel (Laemmli, 1970) and electroblotted onto a nylon filter (Immobilon PVDF, Millipore, Billerica, MA, USA) with a Semi-Dry transfer cell (Bio-Rad). After blocking with 3% BSA-5% nonfat milk-0.06% Tween 20 in PBS for 4hrs, the filter was treated successively with primary (anti-vChti-1, Hiramatsu *et al.*, 1999]) and secondary (alkaline phosphatase-conjugated sheep anti-mouse immunoglobulin G, Boehringer Mannheim) antibodies for 1hrs, at room temperature in 3% BSA-0.06% Tween 20 in PBS. The blot was rinsed twice with 20 mM Tris-HCl (pH 9.0) and reacted with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Detection of chitinase activity after SDS-PAGE

SDS-PAGE used a 10% polyacrylamide gel containing 0.01% ethyleneglycol chitin (Seikagaku Corp.) as substrate. To retain enzyme activities, protein samples were denatured without β-mercaptoethanol at 60 °C for 2 min. After electrophoresis, the gel was incubated in 200 ml of sodium acetate buffer (0.1 M at pH 5.0) containing 1% Triton X-100 at room temperature for 1hrs. The buffer was replaced with a fresh one without Triton X-100, and the gel was incubated overnight at 28 °C with gentle shaking. Chitinase activity was detected by staining the gel with Calcofluor white M2R (Sigma-Aldrich Japan KK, Tokyo) as described by Trudel and Asselin, 1989. Destained and lytic zones in the gel were photographed.

RESULTS

Detection of a *Chlorella* chitinase gene

In our previous work, many immediate-early expressed genes of *Chlorella* viruses were detected and characterized (Kawasaki *et al.*, 2004). These genes (clones) were screened from a cDNA library constructed with poly(A)-containing RNAs extracted from *Chlorella* virus CVK2-infected *Chlorella* sp. NC64A cells at 10 min postinfection (p.i.). Although most of the obtained clones showed nucleotide sequence identity with the genome of *Chlorella* virus represented by PBCV-1 (Van Etten and Meints, 1999), a few clones were apparently different from viral genes. Such a clone, #44 contained an insert of approximately 2.0 kbp, whose sequence showed some homology with the *vChti-1* gene of CVK2. The *vChti-1* gene consists of 2,508 bp and encodes a 836 aa polypeptide containing two sets of catalytic domains of family 18 glycosyl hydrolases (chitinases) (Hiramatsu *et al.*, 1999, Hiramatsu *et al.*, 2000). However, *vChti-1* is a late gene of the virus and shown to be expressed after 100-120 min p.i. (Hiramatsu *et al.*, 1999), which raised a question about the origin and expression pattern of clone #44.

To see an exact expression pattern of clone #44, Northern hybridization was performed where total RNA isolated from CVK2-infected *Chlorella* NC64A cells at various times postinfection was probed with labeled #44 clone DNA. The result shown in Fig. 1A revealed a transcript of approximately 2.7 kb appeared at 0 mim p.i. and persisted till up to 120 min p.i. This result suggested that the #44 transcript was not from a virus gene but expressed from a host gene, probably before virus infection. By some mechanisms, it lasted long time after the breakdown of host gene expression (Van Etten, 2003).

The host origin of clone #44 was confirmed by Southern blot hybridization of genomic DNA isolated from *Chlorella* strains. When *Sph*I digested genomic DNA of *Chlorella* NC64A was hybridized with #44 DNA probe (1.0 kbp *Kpn*I fragment) as a probe , a 6.0 kbp positive band appeared (Fig. 1B). Genomic DNA of virus-sensitive strains 211-6 and F36-ZK also showed similar hybridization bands, whereas genomic DNA of virus-resistant strains *C. ellipsoidea* C-87 and *C. vulgaris* C-169 did not show any hybridizing bands (Fig. 1B). Similar results were observed when genomic DNA was digested with other restriction enzymes; always virus-sensitive strains NC64A, 211-6, and F36-ZK gave positive signals and virus-resistant strains C-87 and C-169 gave no

signal (data not shown). These results indicated that the gene for clone #44 was encoded by host genomic DNA and specifically conserved in virus-sensitive *Chlorella* strains.

Fig.(1A)

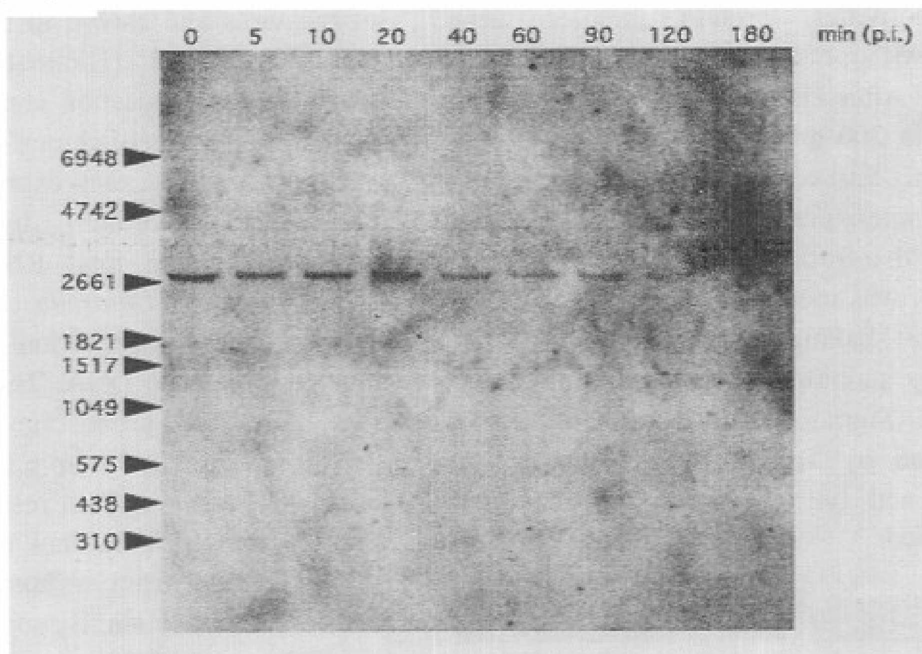

Fig.(1B)

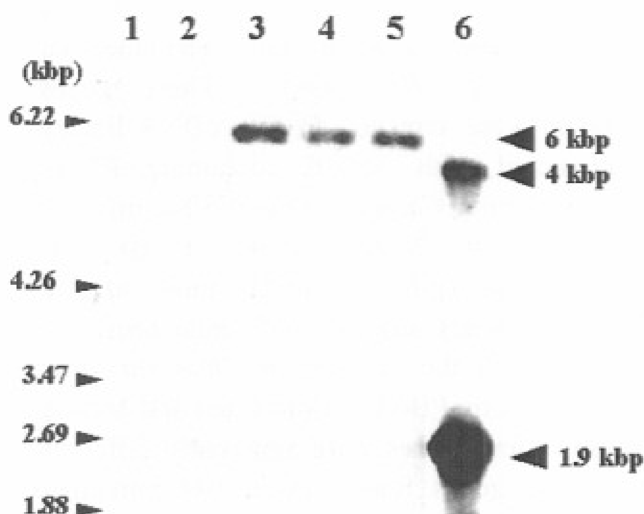

Fig. (1): Detection of a Chlorella chitinase gene. (A) Northern blot hybridization of RNAs isolated from Chlorella strain NC64A cells infected with Chlorella virus CVK2 at various timens postinfection (p.i.). The blot was hybridized with ^{32}P-labeled cDNA clone #44 as a probe. A transcript of approximately 2.7 kb appeared from 0 min p.i. and was retained till 120 min p.i. (B) Southern blot hybridization of Chlorella genomic DNAs with cDNA clone #44 as a probe. Lanes 1 to 5 contained SphI-digested genomic DNA of Chlorella strains C-87, C-169, NC64A, 211-6, and F36-ZK, respectively. Lane 6 contained cDNA clone #44 as a positive control A positive band of approximately 6 kbp in size was seen for virus-sensitive strains, NC64A, 211-6, and F36-ZK.

Characterization of the *Chlorella* chitinase gene, hChti-1

Nucleotide serquence determined for clone #44 DNA revealed a 1,881 bp sequence containing a 5'-truncated ORF of 401 aa, a 669 bp 3'-untranslated region, and 8bp of the poly (A) tail. Homology search through the databases for the ORF sequence showed the highest FASTA score of 965 to vChti-1 of CVK2 (Hiramatsu *et al.*, 1999) or A181/182R of PBCV-1 (Sun *et al.*, 1999). When the amino acid sequence of a chitinase domain (clone #44) was compared with that of vChti-1 by the matrix plot method, both catalytic domains of vChti-1 showed significant homology (Fig. 2C). There was 42% aa identity between #44 sequence and the first catalytic domain (aa positions 1-400) and 34% identity between #44 and the second catalytic domain (aa positions 450-837) of vChti-1. The two regions (regions 1 and 2) in the catalytic domain that are conserved in almost all microbial chitinases (Watanabe *et al.*, 1993) are also conserved in the hChti-1 chitinase domain as shown in Fig. 2B. Two essential aa residues in region 2, Asp and Glu needed for catalytic activity are surely retained in hChti-1, suggesting actual enzymatic activity of hChti-1. Instead of such a significant homology in amino acid sequence between hChti-1 and vChti-1 chjitinase domains, homology at nucleotide sequence level was not so high; approximately 55% nucleotide identity was seen between *hChti-1* and *vCht-1* genes in the regions compared in Fig. 2C. This was mainly caused by differences in the GC-content and codon usage in the host and virus genomes; the GC-contents are 67% and 41% for the host (*Chlorella* strain NC64A) genome (Van Etten *et al.*, 1991) and virus CVK2 genome (Yamada *et al.*, 1991), respectively. The relationship (origin and evolution) between *hChti-1* and *vChti-1* is very interesting.

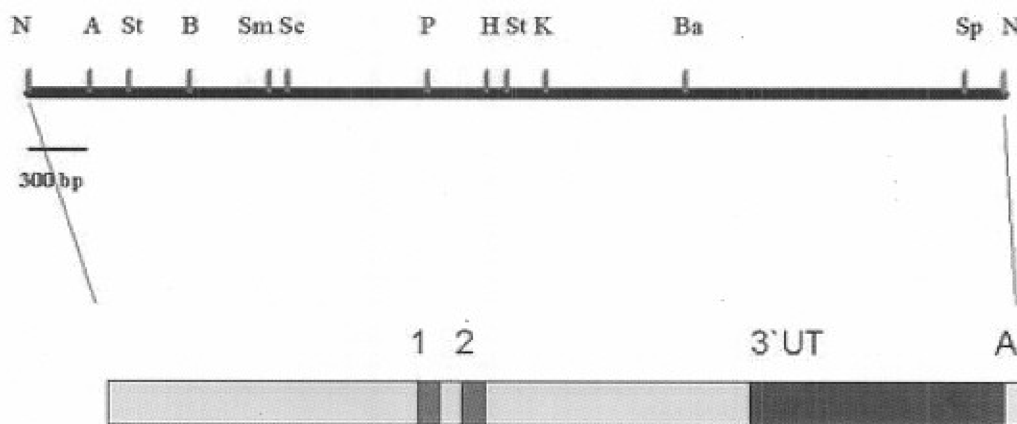

Fig. (2 A): Legend, see next page.

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   1  TCTTTCGAGGCCAGCTTCTCCATCAAGAACTCAAACGCCTTCTACATCCTCAACTGGCGC   60
      S  F  E  A  S  F  S  I  K  N  S  N  A  F  Y  I  L  N  W  R
  61  CTGCACTTCAGCCTGGTGGGCTGGAGCAGCTTCACGTGGGGCCCCTCGGACACAGACCTC  120
      L  H  F  S  L  V  G  W  S  S  F  T  W  G  P  S  D  T  D  L
 121  AAGTACGCGGGCACCAAGACCAATGCGCAGGTGGTGCTGACTCCCAAGGAGTGGCTGCGG  180
      K  Y  A  G  T  K  T  N  A  Q  V  V  L  T  P  K  E  W  L  R
 181  GAGATCCCGCCGGGCACCACCCTGAGCATCAGCTTTGGCGGCCAGGGCGTGGCGCCCTCG  240
      E  I  P  P  G  T  T  L  S  I  S  F  G  G  Q  G  V  A  P  S
 241  GCCATCGTCTTTGAGCAGATCCTGCCGCTGCTGGATCCAGACCATGACATTTCCCTCAGC  300
      A  I  V  F  E  Q  I  L  P  L  L  D  P  D  H  D  I  S  L  S
 301  ACGCGGGGCGCCTTCCCGCAAAAGCTCTTTGCCCCCTTTGTGGACGCCACCCTGTACCCC  360
      T  R  G  A  F  P  Q  K  L  F  A  P  F  V  D  A  T  L  Y  P
 361  ACGCCGCGCCTGCTGGATGCCTACGAGGCCACTGGCCAGAAGTGGTTCACCCTGGCCTTC  420
      T  P  R  L  L  D  A  Y  E  A  T  G  Q  K  W  F  T  L  A  F
 421  ATCACCGCTGACCTGCGCACCGGAGCCCGCCTGGGGCGCGTCATCCCGCTGTGGAAGCAG  480
      I  T  A  D  L  R  T  G  A  R  L  G  R  V  I  P  L  W  K  Q
 481  TACTTCATGGACCAGATCCGGGACATCCGCCTGCTGGGTGGCGACTGCATCGTGTCGTTC  540
      Y  F  M  D  Q  I  R  D  I  R  L  L  G  G  D  C  I  V  S  F
 541  GGCGGCGCGGCGGGGCAGGAGCAGGCGCAGGTCCGTGTGGACGAGGACATGCTGCTCAAG  600
      G  G  A  A  G  Q  E  Q  A  Q  V  R  V  D  E  D  M  L  L  K
 601  GACTACCAGACCATAGTGGACCTGTACAAGCTGCGCTGGATCGACTTTGACATCGAGGGC  660
      D  Y  Q  T  I  V  D  L  Y  K  L  R  W  I  D  F  D  I  E  G
 661  GGCGCCGTGCTGGAGATGGCCTCGGTGCAGCGCCGCCACCGCGTGCTCAAGCGCCTGCAG  720
      G  A  V  L  E  M  A  S  V  Q  R  R  H  R  V  L  K  R  L  Q
 721  GACGCCAACCCAGGCCTCGTCGTCTCCTTCACGCTGCCCGTGCTGCCCGTCGGGCTGACC  780
      D  A  N  P  G  L  V  V  S  F  T  L  P  V  L  P  V  G  L  T
 781  GCCGACGGCGTCAACCTGCTGCGGGATGCCAAGGCCAAGGGCGTGCGCCTGGACGTGCTC  840
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 841  AACATCATGACCATGGACTATGGCGACTCGGCGGCGCCAAACCCCAGAGGCCAGATGGGC  900
      N  I  M  T  M  D  Y  G  D  S  A  A  P  N  P  R  G  Q  M  G
 901  GACTATGCCATCCAGGCCGCCGTCAACACCCGCGCGCAGGCCCAGAGCGTGGGCTATGAC  960
      D  Y  A  I  Q  A  A  V  N  T  R  A  Q  A  Q  S  V  G  Y  D
 961  GACACCAAGATCGGCAACACGCCCATGATTGGGCTGAACGATGTGGAGTCTGAGATTTTC  1020
      D  T  K  I  G  N  T  P  M  I  G  L  N  D  V  E  S  E  I  F
1021  TACCTGGATGATGCGCGCAAGGTCGGCGCCTGGGCCAAGGCCACCCCCTGGGTCTCCATC  1080
      Y  L  D  D  A  R  K  V  G  A  W  A  K  A  T  P  W  V  S  I
1081  CACTCCTCCTCCATCCCCCAGGAGCCGTTTGAGTTCACCCGGATCTTCAAAGTCTCCATC  1140
      H  S  S  S  I  P  Q  E  P  F  E  F  T  R  I  F  K  V  S  I
1141  CACTCCTCCTCCATCCCCCAGGAGCCGTTTGAGTTCACCCGGATCTTCAAATCCTTCGCC  1200
      H  S  S  S  I  P  Q  E  P  F  E  F  T  R  I  F  K  S  F  A
1201  AGCTGAGCCTTGTGGCTGACCCGCGATGACCACAACTGAGCGCAGCTGCACACGGACGAA  1260
      S  *
1261  TAGAATCTATTAGATGGTACCAGCGAGCGCTGAGCTGGCATCACTGAGCTGGCATGGCTA  1320
1321  CACGGCGCGCGCCACCTGACACCAACACTGAACTGAGAGCAGCAGAGCCAGACAACCCAG  1380
1381  CCGTGAGAATAGATAGCTACTGTGCACTTCATTTTGGTAGGCGCCAGCATTCAAAAGAGC  1440
1441  CCCCCAGCACTGCCCCCCGCACTGCCGCCCTTTTTCCGCCCGCCGCGGGCAAAATTTATT  1500
1501  CCTAGATTGGTTTCTTCTCCTCCATTCCTTCTCCCGTCCGATCCCCATCTTGATTTGTAC  1560
1561  ATTCCGTCCCTCAGCCCTGCCATCCAATTCACCCCCTATTTTTCCTGACTGAGAGTTACT  1620
1621  TGCATGGATGGTCGCCCATTCCGAGTGCTGCCAACCTCGTGTCCCTTGGCCTGAGCGTCG  1680
1681  CCTGGCAGCGCTCGCACACATGTTTCCCCTTGCGCAAAAAGCGTGGCCTCCTGCGGCTGT  1740
1741  GATTGCTGTGCGGCCCCATGGATAGCCGGCCAGCCAGCAAATGCATGCCCTAGTGCGCCC  1800
1801  CCAACCCCAAACTGTGTGACTGCGACATTGCCGCTGCAAGCATGCACAATCGCTTTTTGT  1860
1861  AACGAGCGACTGACCAAAAAA                                         1881
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Fig.(2 B): Legend, see next page.

Fig.(2-c)

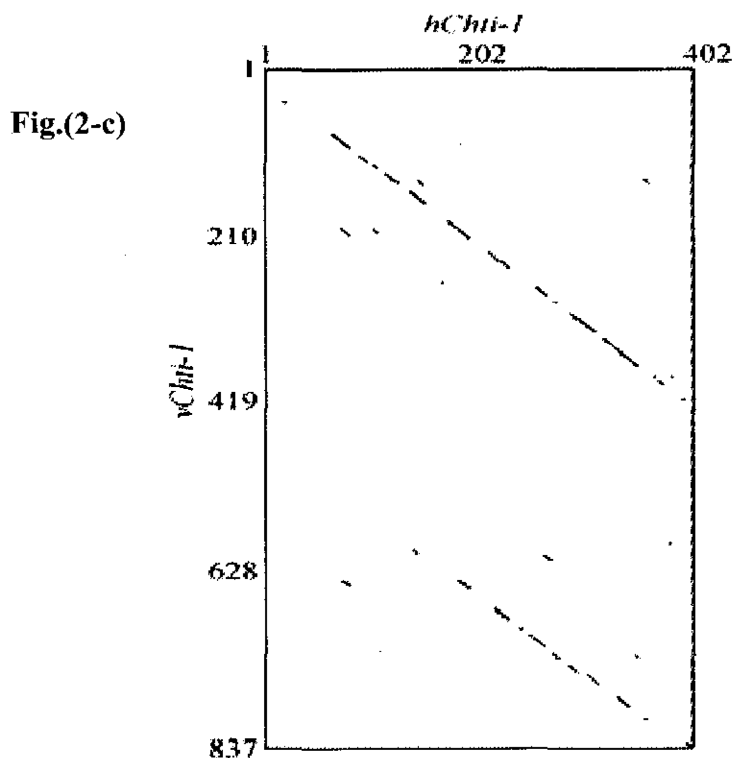

Fig. (2): Characterization of the Chlorella chitinase gene, hChti-1. (A) Physical map of hChti-1 and cDNA clone #44. Clone #44 was screened from a cDNA library (25) and found to be a 5'-truncated clone of hChti-1. The hChti-1 ORF, 3'-untranslated region (3'UT), and a poly (A) tail (poly A) are indicated by separate boxes. The conserved elements 1 and 2 in the catalytic domain of family 18 glycosyl hydrolase are indicated in the ORF. Restriction sites: A, ApaI; B, BamHI; Ba, BanII; H, HincII; K, KpnI; N, NotI; P, PstI; Sc, ScaI; Sm, SmaI; Sp, SphI; St, StyI. (B) Nucleotide and deduced amino acid sequences of hChti-1. The amino acid residues essential for chitinase activity and conserved amino acid sequence elements 1 and 2 in the catalytic domain of family 18 glycosyl hydrolases are shown in blue and red, respectively. (C) Matrix comparison of the amino acid sequence between hChti-1 ORF (positions 1-401) and CVK2 vChti-1. More than 5 matches between 10 amino acid sequences are marked by dots (DNASIS). The conserved sequence elements 1 and 2 within the catalytic domain of family 18 glycosyl hydrolases (15) are shown by arrows.

Expression of *hChti-1* gene in the host cells under stressed conditions

As described above, *hChti-1* gene was detected as an immediate early gene expressed in virus-infected *Chlorella* cells. However, the expression signals were detected at 0 time p.i. (Fig. 1A), raised a question about its expression patterns in *Chlorella* cells. In most plant cells, chitinases are involved in defence mechanisms and their expression is induced under various stressed conditions (Collinge *et al*., 1993). To see *hChti-1* expression patterns, *hChti-1* mRNA levels were quantitatively estimated by real-time PCR method: Total RNA was isolated from *Chlorella* strain NC64A cells incubated under different stressed conditions were subjected to quantitative real-time PCR as described in Materials and Methods. Stressed conditions included different incubation temperatures from 9 °C to 40 °C, different pH of the medium from pH 2 to 10, high salt concentration (0.5 M NaCl), and complete darkness, treatment with chitinase or cellulase, and CVK2 infection. In each case, quantification of alpha-tubulin mRNA levels was paralleled and used to normalize *hChti-1* expression levels. As shown in Fig. 3, expression of *hChti-1* was specifically induced by CVK2 infection; 10 times higher level of mRNA was accumulated in the cells. Chitinase treatment also enhanced the *hChti-1* expression; the expression increased to a four times higher level, whereas treatment with cellulase had no effect on the *hChti-1* levels. In the previous studies, involvement of chitinase and chitosanase activities associated with virus particles in the virus infection process was demonstrated (Hiramatsu *et al*., 1999; Sun *et al*., 1999; Songsri *et al*., 1997), and so the hChti-1 expression seemed to be highly related to virus infection.

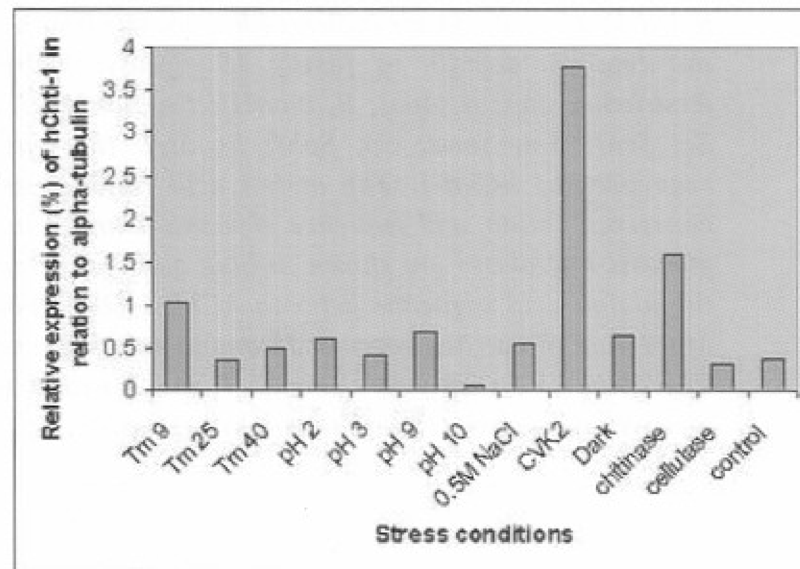

Fig.(3): Expression of the hChti-1 gene in Chlorella NC64A cells under stressed conditions determined by quantitative real-time PCR experiments. In each case, quantification of alpha-tubulin mRNA levels was paralleled and used to normalize hChti-1 expression levels. Ten times higher level of mRNA was accumulated in the cells infected with CVK2.

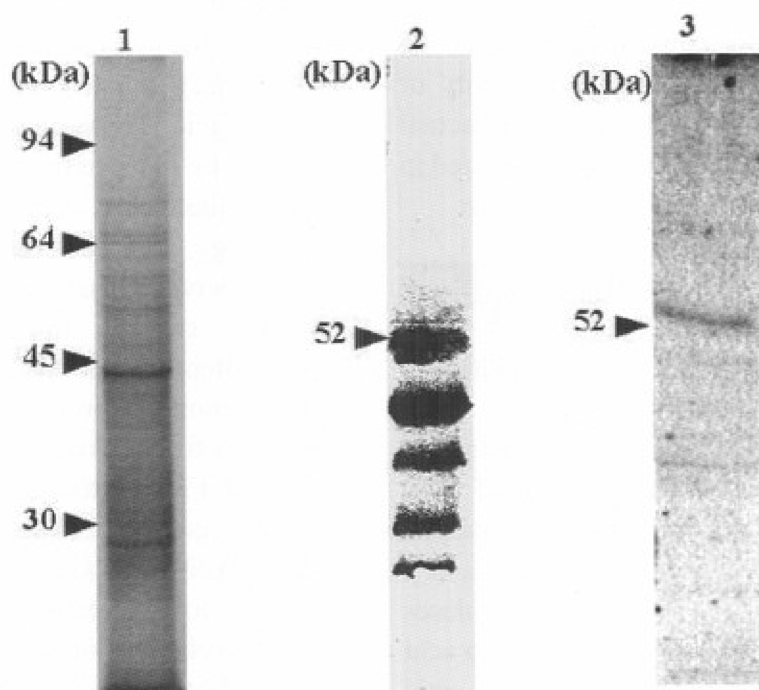

Fig.(4): Detection of hChti-1 protein by Western blot analysis and hChti-1 chitinase activity by zymogram. Total proteins of Chlorella strain NC64A cells were separated by SDS-PAGE and stained with CBB dye (lane 1), treated with anti-vChti-1 antibody in Western blot analysis (lane 2), and subjected to zymogram with ethyleneglycol chitin as a substrate (lane 3). A band corresponding to the expected size 52 kDa was detected in both lanes 2 and 3.

Demonstration of the hChti-1 protein synthesis and chitinase activity in *Chlorella* cells

To see whether enzymatically active hChti-1 protein is produced in *Chlorella* cells, Western blot analysis and zymogram assays were performed. Total proteins were prepared from *Chlorella* NC64A cells grown in MBBM containing ethyleneglycol chitin as an inducer. Because the amino acid sequence of hChti-1 chitinase domain showed high homology with that of vChti-1, we expected that antibody against vChti-1 which we prepared previously (Hiramatsu *et al*., 1999) would react with hChti-1. When NC64A proteins were subjected to Western blot analysis with anti-vChti-1 antibody, several reacting bands were detected as shown in Fig. 4 (lane 2). The largest band coresoponded to a size of 52 kDa, almost the predicted size of hChti-1 protein from the nucleotide sequence. In a control experiment where total protein of *C. vulgaris* C-169 was applied, no reacting signals appeared. These results showed that hChti-1 protein was actually produced in *Chlorella* strain NC64A cells. The nature of the smaller bands was not clear, possibly processing or degradation products in the cells. hChti-1 proteins produced in the *Chlorella* cells were shown to be enzymatically active by

zymogram as indicated in Fig. 4 (lane 3), where ethyleneglycol chitin embedded in the gel was digested by the chitinase activity of proteins. The 54 kDa protein showed actual chitinase activity and smaller bands detected in Western blot experiments were also active.

DISCUSSION

Relationship between *Chlorella* chitinase hChti-1 and virus chitinase vChti-1

In our previous work, a characteristic feature of the virus chitinase vChti-1 was shown to be a duplication of the catalytic domains containing conserved region 1 and 2 of the family 18 glycosyl hydrolases (Henrissat and Bairoch, 1993). The amino acid sequence of the first domain (amino acid positions 144-413) is most similar to the catalytic sequence of *Saccharopolyspora* (*Streptomyces*) *erythraeus* (30% identity), whereas the second domain (positions 560-836) resembles that of *Ewingella americana* (34.7% identity, Hiramatsu *et al.*, 1999). These domains are connected by repeated sequences of amino acids rich in proline residues, like linker structures that are often seen in glycosyl hydrolases to connect domains of different functions (Hiramatsu *et al.*, 1999). Therefore, the two catalytic domains of vChti-1 protein seem not to occur by a simple duplication of a single domain but by connecting two of different origins. The first domain of vChti-1 showed only exochitinase activity and lacked in N-acetylglucosaminidase activity, whereas the second domain contained the N-acetylglucosaminidase activity. Such a composite structure of vChti-1 raised a question about not only its biological function but the origin and evolution of vChti-1. In this work, we accidentally found a host (*Chlorella*)-encoded chitinase (hChti-1). The amino acid sequence of hChti-1 was most similar to the catalytic domain 1 of vChti-1 (42% identical). However, no significant homology was seen at nucleotide sequence levels between their encoding genes. This is mainly caused by differences in the GC-content and codon usage in the host and virus genomes; the total GC-contents are 67% and 41% for the host (*Chlorella* strain NC64A) genome (Van Etten *et al.*, 1991) and virus CVK2 genome (Yamada *et al.*, 1991), respectively. The GC contents of *hChti-1* and vChti-1 coding regions are 64.8% and 54%, respectively. It is interesting to note that genes encoded on *Chlorella* genomes always contain numerous introns; for example, genes for nitrate reductase and alpha-tubulin of *C. vulgaris* contained 18 and 9 introns, respectively (Maki *et al.*, 2000). Contrasting to this, *hChti-1* does not seem to contain introns because PCR experiments with 5'- and 3'-end sequences of *hChti-1* ORF (Fig. 2A) as primers gave products from the genomic DNA of exact by the same size as cDNA (data not shown). This suggests an unusual situation of the gene in the *Chlorella* genome.

In this work, the *hChti-1* gene was detected in three strains of *Chlorella*, all of which are sensitive to *Chlorella* virus infection. All other virus-resistant strains (only a few strains were shown in this work) did not contain the gene on the genome. Recently, a phylogenetically close relationship among the three strains (NC64A, 2116, and F36-ZK) was suggested by comparison of rDNA sequences (Hoshina *et al.*, 2004; T. Yamada, unpublished result). The 18S rRNA gene of these strains contained three group-I self-splicing introns at

the same sites and the nucleotide sequence of 18S rDNA was the same among the three. Similar self-splicing group-I introns were also reported in the genes of *Chlorella* viruses (Yamada *et al.*, 1994, Nishida *et al.*, 1998). These results, altogether suggest that the *vChti-1* gene might have been horizontally transmitted between a specific strain of *Chlorella* and viruses.

Properties and functions of *hChti-1* chitinase

The amino acid sequence of *hChti-1* showed high homology with catalytic domain I of vChti-1 and retained two conserved elements and essential amino acid residues of family 18 chitinases (Fig. 2B), suggesting very similar activities to that demonstrated for the first domain of vChti-1 (Hiramatsu *et al.*, 2000) and *S. erythraeus* chitinase (Hara *et al.*, 1989). In fact, hChti-1 protein expressed in *Chlorella* NC64A cells showed a chitinase activity (Fig. 4). Several bands smaller than the expected size also appeared in western blot and zymogram analyses and they may be intermediates of processing or degradation. Chitinase activity of hChti-1 could also be detected by a halo-forming test where colonies of strain NC64A were formed on MBBM plates containing 0.01% ethyleneglycol chitin as substrate. Clear halo was visible around the colonies after staining with Calcofluor White M2R dye (data not shown), suggesting that hChti-1 proteins were secreted from the cells into the medium.

Induced expression of chitinase and other proteins in cells of higher plants by microbial infection, wounding, and plant stress hormones has been implicated as a defence mechanism against pathogen infection (Collinge *et al.*, 1993). Targets of such plant chitinase activities are cell surface materials of bacterial or fungal pathogens or insects. In the case of the multicellular green alga *Volvox*, a chitinase gene was induced to be expressed by sex-pheromone and wounding (Amon *et al.*, 1998). This is only one other example of algal chitinase known to date but the enzyme target is completely unknown. Chitin or chitin-like substances have never observed as constituents of extracellular materials of most green algae including the order Volvocales. However, chitin-like macromolecules have exceptionally been identified as cell wall components in certain strains of *Chlorella* (Kapaum and Reisser, 1995). *Chlorella* strains sensitive to virus such as those studied in this work contained glucosamine as a cell wall constituent and are supposed to have chitin-like materials in the cell wall (Chuchird *et al.*, 2001). In such *Chlorella* cells, a chitinase should function to modify the cell wall during cell growth and cell division. It may be a good strategy for viruses to utilize such an enzyme to break the cell wall barrier during infection. In fact, chtinase and chitosanase proteins are embedded in virus particles and were suggested to digest the host cell wall at the virus attachment site (Hiramatsu *et al.*, 1999; Hiranmatsu *et al.*, 2000; Songsri *et al.*, 1997). In this work, it was shown that the expression of hChti-1 was induced by virus infection and chitinase-treatment (Fig. 3). A possible explanation for this response is not by a defense mechanism as observed in higher plants but by a regulation of the gene expression including chitooligosaccharides as an inducer. Such materials can be formed from the cell wall by virus infection (by digestion with virus enzymes) as well as by chitinase treatment.

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الملخص العربي

وصف جين الكيتينيز المكون بواسطة الأنواع القابلة للإصابة من طحلب الكلوريلا أثناء الإصابة بفيروس الكلوريل

على محمد محمد على و تاكيرو كاوازاكى و تاكاشى يامادا

قسم البيوتكنولوجيا الحيوية- المدرسه العليا للعلوم المتقدمة-جامعة هيروشيما- اليابان

عند دراسة العلاقة بين الفيروسات التى تصيب طحلب الكلوريلا وخلية الطحلب أمكن عزل ودراسة جين الكيتينيز المسمى (hChti-1).أوضحت النتائج أن الجزء الذى تم عزله من الجين يحتوى على ٤٠١ حمض أمينى وأن هذه الأحماض الأمينية ذات تشابه مع الجزء الأول من جين الكيتينيز المعزول من فيروس الكلوريلا المسمى (CVK2) والذى يقع تحت العائلة ١٨ من عائلات الكيتينيز. كذلك أوضحت الدراسة أن جين الكيتينيز (hChti-1) يتم إستحثاثه أثناء الإصابة بالفيروس, والذى يؤدى إلى تكون البروتين الفعال للجين. وجد أن هذا الجين يتكون فقط فى الأنواع القابلة للإصابة من طحلب الكلوريلا ولا يتكون فى الأنواع المقاومة مما يدل على أهمية هذا الجين فى ميكانيكية الإصابة بالفيروس. هذا التقرير يعتبر الأول فى عزل ودراسة جين الكيتينيز المكون بواسطة الطحالب وحيدة الخلية.