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Expression of a Chitinase Gene and Lysis of the Host Cell Wall during *Chlorella* Virus CVK2 Infection

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A chitinase gene (*vChti-1*) encoded by the *Chlorella* virus CVK2 was cloned and characterized. The *vChti-1* open reading frame consisted of 2508 bp corresponding to 836 amino acid residues. The predicted amino acid sequence contained two sets of a family 18 catalytic domain that is responsible for chitinase activity. Northern blot analysis revealed that the *vChti-1* gene was expressed in virus-infected *Chlorella* cells late in infection, when a single transcript of about 2.5 kb appeared at 120 min postinfection. This result was confirmed by Western blotting with a specific anti-vChti-1 protein antibody; a protein of about 94 kDa was detected specifically beginning at 240 min postinfection and was present until cell lysis. The protein was not incorporated into viral particles but remained in the medium after cell lysis. The vChti-1 protein produced in virus-infected cells showed chitinase activity on zymogram assays.

INTRODUCTION

Large icosahedral, double-stranded DNA-containing viruses that infect certain strains of the unicellular green alga *Chlorella* are ubiquitous in natural environments (for a review, see Van Etten *et al.,* 1991). In a typical lytic cycle of *Paramecium bursaria Chlorella* virus (PBCV-1), the prototype of the Phycodnaviridae (Francki *et al.,* 1991), with *Chlorella* strain NC64A as a host, virus particles attach to the surface of host cells and degrade the cell wall at the point of attachment, and the viral core is released into the host cytoplasm, leaving an empty capsid on the cell wall. Viral DNA synthesis commences by approximately 1 h post infection (p.i.); by 2–3 h p.i., components of the viral capsid accumulate in the cytoplasm. Mature, DNA-filled virions appear by 4 h p.i. and exit the cells after cell lysis within 6–8 h p.i. Although both the initial and final stages of the viral replication cycle require cell wall-digesting activities, very little is known about the nature and origin of the enzymes that digest the cell wall.

A characteristic feature common to virus-sensitive *Chlorella* strains is a rigid cell wall containing glucosamine in addition to other sugars; glucosamine comprises 7–17% of total sugars in the cell wall (Meints *et al.,* 1988; Kapaun *et al.,* 1992; Kapaun and Reisser, 1995; Takeda, 1995). This suggests that enzymes degrading polymers of glucosamine, like chitin (β-1,4-linked polymer of *N*-acetyl-D-glucosamine) and chitosan (β-1,4-linked polymer of D-glucosamine with various degrees of *N*-acetylation), may be involved in the viral infection process.

In an earlier work (Yamada *et al.,* 1997), we demonstrated that the *Chlorella* virus gene, *vChta-1*, in strain CVK2 produces two functional chitosanase proteins with apparently distinct roles in viral infection: The larger 65-kDa chitosanase is assembled into the virion and presumably functions at the beginning of infection, whereas the smaller 37-kDa enzyme remains in the host cytoplasm, where it most likely aids in the digestion of the host cell wall before viral release. However, the vChta-1 chitosanase alone could not digest the host cell wall completely (Yamada *et al.,* 1997), indicating that some additional polysaccharide-degrading activities may be needed to digest the host cell wall. Such presumed activities may include virus-encoded chitinases (Lu *et al.,* 1996) and/or β-1,3-glucanase (Li *et al.,* 1995).

In the present work, we studied expression of a functional chitinase gene (*vChti-1*) that is encoded by the CVK2 genome, and we discuss its origin and possible roles in the viral infection process.

RESULTS

Cloning and sequence analysis of the chitinase gene of CVK2

Lu *et al.* (1996) reported that two chitinase-like ORFs, A181R and A182R, are encoded on the PBCV-1 genome: A181R, which codes for 475 amino acids (aa) and A182R, encoding 230 aa, share 40% aa identity in their 220 carboxyl-terminal aa. The two open reading frames (ORFs) are adjacent in the same orientation and separated by 373 nucleotides on the PBCV-1 DNA. We are

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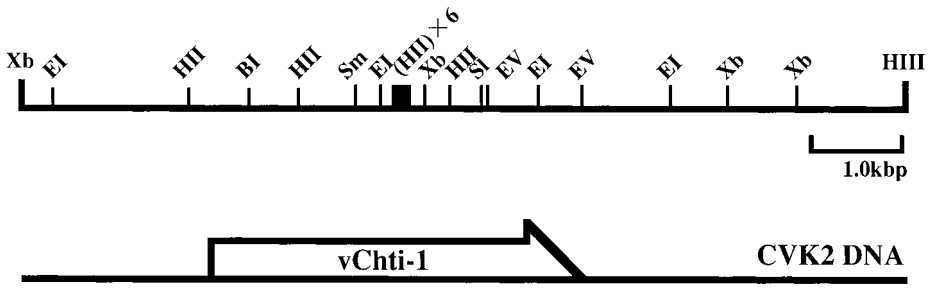

FIG. 1. Physical map of CVK2 genomic DNA (clone 6F3) showing the region encoding the *vChti-1* gene (arrow). Size is shown in kilobase-pairs (kbp). Restriction sites: BI, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; HII, *Hin*cII; HIII, *Hin*dIII; SI, *Sal*I; Sm, *Sma*I; Xb, *Xba*I.

interested in the structure and expression of the corresponding gene(s) of CVK2. Based on the PBCV-1 nucleotide sequence, we synthesized PCR primers; the first 25 nucleotides of the A181R coding region for a forward primer and the last 25 nucleotides of the A182R coding region for a reverse primer. An approximately 2.5-kbp fragment was amplified by PCR with CVK2 DNA as template. The complete chitinase gene (*vChti-1*) and its 5′- and 3′-flanking regions were screened from a CVK2 genomic library (Yamada *et al.,* 1996) by using plaque hybridization with the above PCR product as a probe. A resulting genomic clone (6F3) gave the restriction map shown in Fig. 1. The nucleotide sequence determined for the coding and 5′- and 3′-flanking regions showed that there is a single large ORF of 836 aa instead of two split ORFs such as A181R and A182R. This was caused by an insertion of a G in PBCV-1 at a nucleotide position between 93152 and 93153 and another insertion of C at a position between 93315 and 93316, which resulted in an in-frame connection of the two ORFs of PBCV-1 in the CVK2 sequence. In addition, a 12-bp stretch, 5′-CCTACCAATCCT (PBCV-1 positions 93208–93219), is absent in the CVK2 sequence and a 30-bp sequence, 5′-GTTGACCCTAAACCTGTTGACCCTAAGCCG, is inserted in the CVK2 sequence between positions corresponding to PBCV-1 93470 and 93471. The predicted aa sequence of vChti-1 protein is compared with those of A181R and A182R of PBCV-1 in Fig. 2A. In addition to several aa substitutions in the coding regions (98% identity), major differences exist between the CVK2 and PBCV-1 sequences: Due to a frameshift at position 442 aa of A181R, a region rich in Pro residues is extended in the vChti-1 sequence (Fig. 2A, wavy line) and a 131-aa spacer connected A181R and A182R in vChti-1. This connector region contains six repetitions of a PVDPK motif in vChti-1 (Fig. 2A, arrows). Because the 220 carboxyl-terminal aa of A182R are also conserved in A181R (Lu *et al.,* 1996), vChti-1 protein consequently possesses two sets of them. It is noteworthy that this conserved region corresponds to the catalytic domain of the family 18 glycosyl hydrolases (Henrissat and Bairoch, 1993). According to Watanabe *et al.* (1993), who further divided prokaryotic chitinases of family 18 into three subgroups on the basis of their sequence characteristics, vChti-1 protein can be classified into subgroup C where obvious homology to either *Bacillus circulans* chitinase A1 or D is absent. The two small 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 vChti-1 protein (Fig. 2A). The essential aa residues, Asp and Glu, that are needed for catalytic activity in region 2 are surely retained in the vChti-1 chitinase. The two sets of these regions are linked by a repeated sequence of the aa PVDPK. There is no region that shows obvious homology to fibronectin type III (Watanabe *et al.,* 1993). A model to represent the molecular arrangement of vChti-1 protein is schematically shown in Fig. 2B.

When the upstream flanking nucleotide sequences were compared between *vChti-1* and A181R, a sequence of at least 125 bp that is immediately upstream of the ORF was found to be highly homologous (93.6% identical). This region contained a 65-bp AT-rich (83% AT) sequence with the consensus feature of *Chlorella* virus gene promoters. Within this region, there also were a possible −10 sequence (5′-TATAAA) and a −35 sequence (5′-TTGAAA) with 17-bp spacing resembling *Escherichia coli* promoter elements. As for the downstream region, the nucleotide sequence immediately 3′ to the ORF was also highly homologous with *vChti-1* and A182R; for a stretch of at least 100 bp, it was 99% identical (DDBJ accession no. AB022343).

Expression of the *vChti-1* gene during viral infection

To determine when the *vChti-1* gene is transcribed in the course of viral infection, total RNA was extracted at various times after CVK2 infection and analyzed by Northern blotting using the *vChti-1* gene as probe. The result shown in Fig. 3 indicates that a single transcript of approximately 2.5 kb first appears at 120 min p.i. (lane 4) and remains up to 360 min p.i. The size of the transcript was the same as that of a single ORF for *vChti-1*. The transcript was absent early in infection (lanes 1–3), indicating that the *vChti-1* gene is a late gene and that there are no homologous transcripts in host cells before infection. The *vChti-1* expression pattern is, however, a little

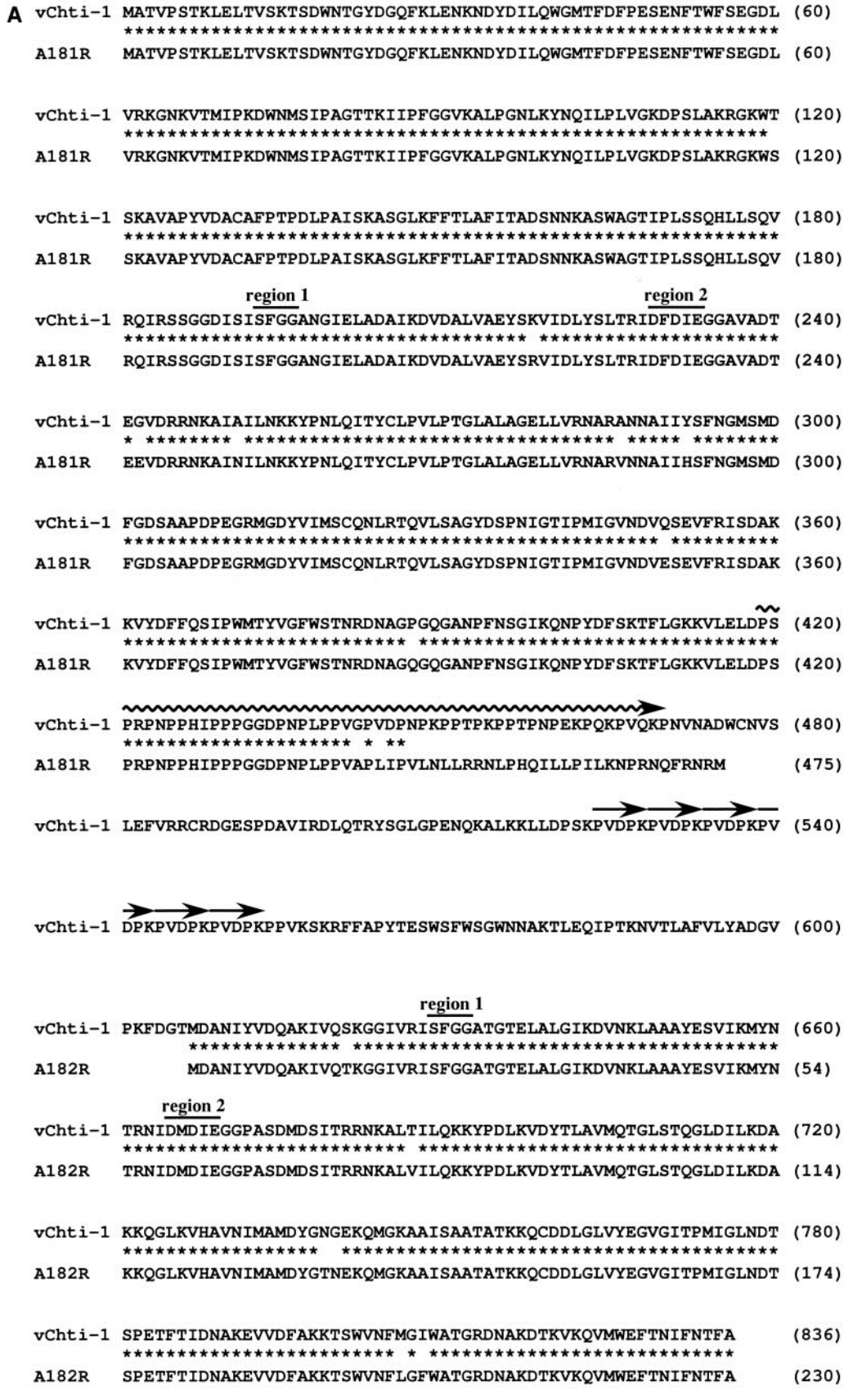
A
vChti-1 MATVPSTKLELTVSKTSDWNTGYDGQFKLENKNDYDILQWGMTFDFPESENFTWFSEGDL (60)
A181R MATVPSTKLELTVSKTSDWNTGYDGQFKLENKNDYDILQWGMTFDFPESENFTWFSEGDL (60)
vChti-1 VRKGNKVTMIPKDWNMSIPAGTTKIIPFGGVKALPGNLKYNQILPLVGKDPSLAKRGKWT (120)
A181R VRKGNKVTMIPKDWNMSIPAGTTKIIPFGGVKALPGNLKYNQILPLVGKDPSLAKRGKWS (120)
vChti-1 SKAVAPYVDACAFPTPDLPAISKASGLKFFTLAFITADSNNKASWAGTIPLSSQHLLSQV (180)
A181R SKAVAPYVDACAFPTPDLPAISKASGLKFFTLAFITADSNNKASWAGTIPLSSQHLLSQV (180)
region 1
region 2
vChti-1 RQIRSSGGDISISFGGANGIELADAIKDVDALVAEYSKVIDLYSLTRIDFDIEGGAVADT (240)
A181R RQIRSSGGDISISFGGANGIELADAIKDVDALVAEYSRVIDLYSLTRIDFDIEGGAVADT (240)
vChti-1 EGVDRRNKAIAILNKKYPNLQITYCLPVLPTGLALAGELLVRNARANNAIIYSFNGMSMD (300)
A181R EEVDRRNKAINILNKKYPNLQITYCLPVLPTGLALAGELLVRNARVNNAIIHSFNGMSMD (300)
vChti-1 FGDSAAPDPEGRMGDYVIMSCQNLRTQVLSAGYDSPNIGTIPMIGVNDVQSEVFRISDAK (360)
A181R FGDSAAPDPEGRMGDYVIMSCQNLRTQVLSAGYDSPNIGTIPMIGVNDVESEVFRISDAK (360)
vChti-1 KVYDFFQSIPWMTYVGFWSTNRDNAGPGQGANPFNSGIKQNPYDFSKTFLGKKVLELDPS (420)
A181R KVYDFFQSIPWMTYVGFWSTNRDNAGQGQGANPFNSGIKQNPYDFSKTFLGKKVLELDPS (420)
vChti-1 PRPNPPHIPPPGGDPNPLPPVGPVDPNPKPPTPKPPTPNPEKPQKPVQKPNVNADWCNVS (480)
A181R PRPNPPHIPPPGGDPNPLPPVAPLIPVLNLLRRNLPHQILLPILKNPRNQFRNRM (475)
vChti-1 LEFVRRCRDGESPDAVIRDLQTRYSGLGPENQKALKKLLDPSKPVDPKPVDPKPVDPKPV (540)
vChti-1 DPKPVDPKPVDPKPPVKSKRFFAPYTESWSFWSGWNNAKTLEQIPTKNVTLAFVLYADGV (600)
region 1
vChti-1 PKFDGTMDANIYVDQAKIVQSKGGIVRISFGGATGTELALGIKDVNKLAAAYESVIKMYN (660)
A182R MDANIYVDQAKIVQTKGGIVRISFGGATGTELALGIKDVNKLAAAYESVIKMYN (54)
region 2
vChti-1 TRNIDMDIEGGPASDMDSITRRNKALTILQKKYPDLKVDYTLAVMQTGLSTQGLDILKDA (720)
A182R TRNIDMDIEGGPASDMDSITRRNKALVILQKKYPDLKVDYTLAVMQTGLSTQGLDILKDA (114)
vChti-1 KKQGLKVHAVNIMAMDYGNGEKQMGKAAISAATATKKQCDDLGLVYEGVGITPMIGLNDT (780)
A182R KKQGLKVHAVNIMAMDYGTNEKQMGKAAISAATATKKQCDDLGLVYEGVGITPMIGLNDT (174)
vChti-1 SPETFTIDNAKEVVDFAKKTSWVNFMGIWATGRDNAKDTKVKQVMWEFTNIFNTFA (836)
A182R SPETFTIDNAKEVVDFAKKTSWVNFLGFWATGRDNAKDTKVKQVMWEFTNIFNTFA (230)

FIGURE 2

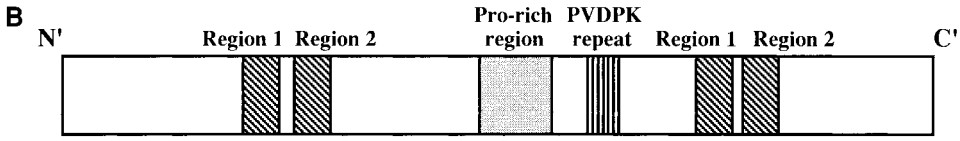

FIG. 2. (A) Comparison of the predicted aa sequence of the CVK2 *vChti-1* gene with those of A181R and A182R of PBCV-1 (Lu *et al.,* 1996). Sequences corresponding to the catalytic regions 1 and 2 of family 18 glycosyl hydrolases (Watanabe *et al.,* 1993) are indicated by lines. Wavy line shows a region rich in Pro residues, and arrows indicate a repetition of a motif of PVDPK. (B) Schematic representation of the molecular arrangement of vChti-1 protein. Regions 1 and 2 conserved in the catalytic domain of family 18 glycosyl hydrolases are indicated by hatched boxes. A Pro-rich region and PVDPK repeats are also shown.

bit different from that of *vChta-1*, the CVK2 gene for chitosanase (Yamada *et al.,* 1997): the former transcript accumulates at 120 min p.i. and gradually decreases in amount, whereas the two vChta-1 transcription products also appear at 120 min p.i., but their quantities gradually increase until the final stage of infection. The molecular basis and the biological significance of the different expression patterns of these genes are very interesting.

Immunological detection of the vChti-1 protein

The production of the vChti-1 protein during CVK2 infection was examined using Western blot analysis. To prepare antibody specific to vChti-1 protein, a glutathione *S*-transferase (GST)–vChti-1 fusion protein was constructed and produced in *E. coli*. The hybrid protein contained the entire vChti-1 region (836 aa) linked to the N′-232 aa and C′-7 aa of the *E. coli* GST. The GST–vChti-1 hybrid protein purified by glutathione–Sepharose 4B affinity chromatography showed a single major band of approximately 120 kDa in SDS–PAGE (Fig. 4, lane 6); the size coincided with the expected one. Anti-vChti-1 antibody was raised in mice against a purified, thrombin-digested fragment of 94 kDa (Fig. 4, lane 8). Total protein of *Chlorella* cells infected by CVK2 was extracted at various times after infection, separated on SDS–PAGE, and subjected to Western blot analysis. The result shown in Fig. 5A indicates that a single protein band of approximately 94 kDa in size is specifically detected at 240 min p.i. (lane 5) and persists to 480 min p.i. (lanes 6 and 7). This result is consistent with the mRNA expression pattern seen in Northern blots (Fig. 3). The size of the product was also in good accordance with that expected for the *vChti-1* ORF. This protein product was not incorporated into the viral particles (lane 8) but was released into the medium after cell lysis (lane 9).

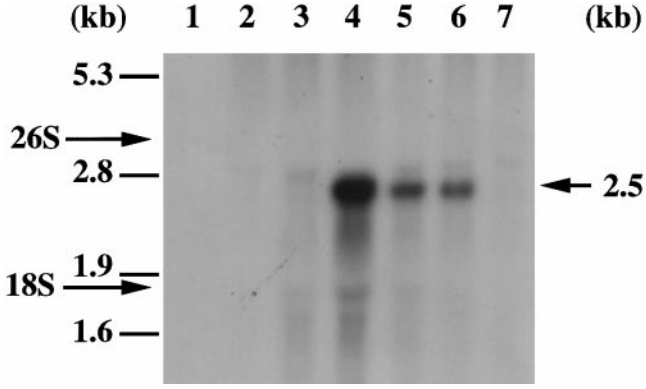

FIG. 3. Northern blot analysis of RNAs isolated from CVK2-infected *Chlorella* cells at various times p.i. Lanes 1–7, total RNA isolated at 0, 20, 60, 120, 240, 360, and 480 min p.i., respectively. The blot was hybridized with ^{32}P-labeled pvChti-1 DNA. Positions of 26S and 18S rRNAs are indicated by arrows.

Chitinase activity of the vChti-1 protein

When total proteins of *Chlorella* cells infected by CVK2 were assayed by zymogram in SDS–PAGE containing glycol chitin as substrate (Trudel and Asselin, 1989), a lytic zone appeared at the position corresponding to the vChti-1 protein (Fig. 5B, lanes 5–7). There was no other band with chitinase activity. Viral particles did not show any discernible chitinase activities (lane 8), whereas the cell lysate contained a major active band of 94 kDa (lane 9). Chitinase activity associated with the CVK2 virion, detected in our previous study (Yamada *et al.,* 1997), was found to be due to contamination with lysate protein; after repeated washing, the active band disappeared in the gel assay. These results suggest that the vChti-1 protein encoded by CVK2 actually expresses its enzymatic activity late in infection and is released into the medium after cell lysis. An approximate activity of 0.029 U/mg protein was detected in the cell lysate. The vChti-1 chitinase did not show lysozyme activity; it could not hydrolyse the β-1,4-linkage between *N*-acetyl muramic acid and *N*-acetyl glucosamine residues in peptidoglycan (data not shown). The catalytic activity of transglycosylation, like that of *Nocardia* chitinase (Usui *et al.,* 1987), was not tested for the vChti-1 chitinase.

DISCUSSION

Structural characteristics of the vChti-1 product

As described for A181R and A182R of PBCV-1 (Lu *et al.,* 1996), the *vChti-1* ORF most resembles chitinases from bacteria and fungi, especially that of *E. coli* (33% aa identity), *Saccharopolyspora* (*Streptomyces*) *erythrae* (29% identity), and *Aeromonas* sp. (29% identity). According to the classification based on aa sequence similari-

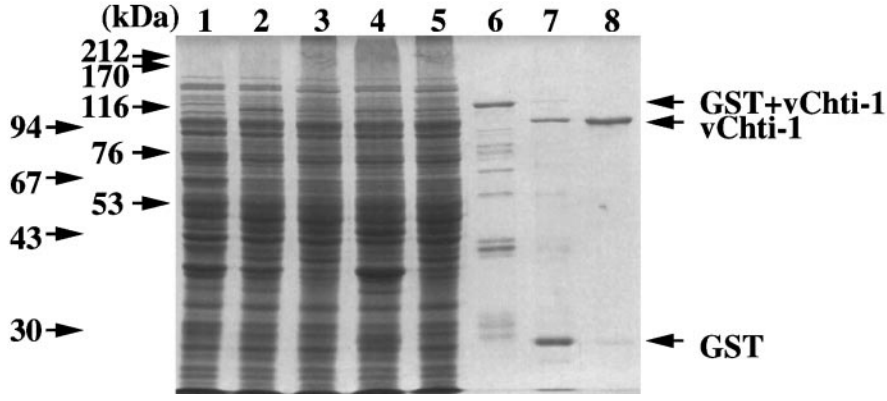

FIG. 4. Production and purification of a GST–vChti-1 fusion protein. Proteins were separated by SDS–PAGE and stained with Coomassie blue. Lane 1, total proteins of *E. coli* BL21 without the plasmid; lane 2, total proteins of *E. coli* BL21 with pGEX-4T-3–vChta-1 after induction with isopropyl-β-D-thiogalactopyranoside; lane 3, soluble fraction after centrifugation of *E. coli* proteins shown in lane 2; lane 4, pellet fraction; lane 5, filtrate fraction through a glutathione–Sepharose affinity column; lane 6, protein purified by affinity chromatography; lane 7, purified protein digested with thrombin; lane 8, purified protein used as antigen.

ties (Henrissat and Bairoch, 1993), these chitinases belong to the family 18 of glycosyl hydrolases, especially to the bacterial subfamily-C of Watanabe *et al.* (1993). A peculiar structural feature of the vChti-1 chitinase is a duplication of possible catalytic domains containing conserved regions 1 and 2 (Watanabe *et al.,* 1993). A chitinase with two catalytic sites has been also reported for the hyperthermophilic archeon *Pyrococcus kodakaraensis* KOD1 (Tanaka *et al.,*1998). In CVK2 vChti-1 chitinase, these domains are connected by repeated sequences of aa rich in Pro (Figs. 2A and 2B). Major differences between CVK2 and PBCV-1 proteins are attributed to the size variation of this linker region. Such Pro- and/or Thr-rich linkers are often observed in glycosyl hydrolases (Gilkes *et al.,* 1991). Aside from the catalytic domains, there are no obvious structural features such as a signal peptide, chitin-binding domains, fibronectin type III-like domains, or a C′-extension. Therefore, vChti-1 chitinase may represent one of the simplest structures to display the catalytic activity.

Chitinase of viral origin is very rare, but recently another example was reported: Hawtin *et al.* (1995) characterized a chitinase gene encoded on the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) genome. Based on phylogenetic analyses, they supposed that the virus acquired the gene from a bacterium such as *Serratia marcescens* via horizontal gene transfer.

In this connection, it is interesting that the aa sequence of the first catalytic domain on vChti-1 protein (positions 144–413) is most similar to the catalytic sequence of *S. erythrae* (30.0% identity), whereas the sec-

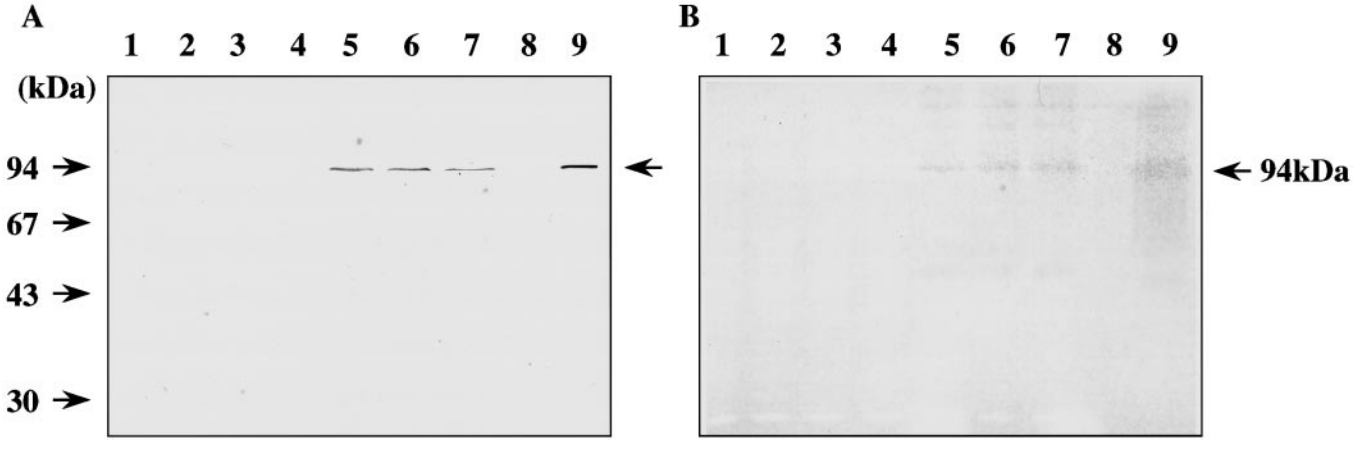

FIG. 5. (A) Western blot of total proteins prepared from CVK2-infected *Chlorella* cells at 0, 20, 60, 120, 240, 360, and 480 min p.i. (lanes 1–7, respectively). Lanes 8 and 9 contained proteins from purified CVK2 particles and *Chlorella* cell lysate separated from which virus particles were removed by centrifugation. The blot was treated with mouse anti-vChti-1 antibody. The antibody detected specifically a single protein band of 94 kDa as indicated by arrow. (B) Detection of chitinase activities in proteins prepared as in A. After electrophoresis in an SDS–10% polyacrylamide gel containing 0.01% glycol chitin as substrate, chitinase activity was detected by staining the gel with Calcofluor dye. Note a major band corresponding to the size of 94 kDa (arrow). Lanes are the same as in A.

ond domain (positions 560–836) resemble that of *Ewingella americana* (34.7% identity) (Fig. 2A).

This result suggests different origins for the two domains of vChti-1 chitinase. The presence of a set of prokaryotic promoter element-like sequences in the upstream region of the *vChti-1* ORF also supports a bacterial origin for this gene.

Biological significance of the CVK2 chitinase

The *vChti-1* product with chitinase activity was produced late in CVK2 infection. In contrast to the *vChta-1* products with chitosanase activity, a larger form of which is incorporated into the virion (Yamada *et al.,* 1997), the vChti-1 protein was not detected in the CVK2 virion (Fig. 5A). Therefore, it does not seem to be involved in the initial events. Instead, as suggested for the smaller form of the vChta-1 chitosanase (Yamada *et al.,* 1997), vChti-1 chitinase remains in the host cytoplasm where it most likely aids in the digestion of the host cell wall before viral release at the final stage of infection. Preliminary experiments, in which isolated host cell–wall materials were treated with the vChti-1 chitinase and/or vChta-1 chitosanase, show the production of some degradation products, supposed to be sugar oligomers (data not shown). *Chlorella* NC64A cells treated with vChti-1 chitinase and vChta-1 chitosanase did not show significant morphological changes, so additional enzymatic activities may be required to efficiently digest the host's complex cell wall (Meints *et al.,* 1988; Kapaun *et al.,* 1992; Kapaun and Reisser, 1995; Takeda, 1995).

The *vChti-1* gene is highly conserved among *Chlorella* viruses; all 50 random isolates of *Chlorella* viruses (Yamada *et al.,* 1993) showed a strong signal in genomic Southern blots with a cloned *vChti-1* DNA as probe (data not shown), indicating the possible importance of this gene. Other potential enzymes encoded by *Chlorella* virus PBCV-1 that might be involved in cell wall digestion include another chitinase encoded by A260R (Lu *et al.,* 1996) and β-1,3-glucanase (A94L) (Li *et al.,* 1995). However, as shown in Fig. 5B, there is no chitinase band at 54 kDa expected for the A94L product, in either virus-infected cells or viral particles. Moreover, the CVK2 genome does not contain the gene for β-1,3-glucanase (T. Yamada, unpublished result), and almost half of the viral isolates so far tested do not possess this gene, as revealed by Southern blotting (data not shown). Host enzymes such as the one used for *Chlorella* protoplast formation by Hatano *et al.* (1992) might be involved in cell lysis.

MATERIALS AND METHODS

Cells and virus

Cells of *Chlorella* strain NC64A (Muscatine *et al.,* 1967) were cultured in a modified Bold's basal medium 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).

The production and purification of CVK2 were performed as described previously (Yamada *et al.,* 1991).

Fractionation of viral proteins

Purified virus particles were treated with 4 M urea and fractionated into the capsid and core fractions as described previously (Yamada *et al.,* 1996). The fractions were subjected to SDS–PAGE according to Laemmli (1970). Molecular size markers LMW kit E and HMW kit E (Pharmacia Biotech) contained rabbit muscle phosphorylase b (94,000 Da), BSA (67,000 Da), rabbit muscle aldolase (43,000 Da), bovine erythrocyte carbonic anhydrase (30,000 Da), myosin (212,000 Da), α_2-macroglobulin (170,000 Da), β-galactosidase (116,000 Da), transferrin (76,000 Da), and glutamate dehydrogenase (53,000 Da).

Detection of chitinase activity after SDS–PAGE

SDS–PAGE used a 10% polyacrylamide gel containing 0.01% glycol chitin (Seikagaku Corp.) as substrate. 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 1 h. 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) as described by Trudel and Asselin (1989). Destained and lytic zones in the gel were photographed.

Chitinase activity was assayed according to Dygert *et al.* (1965). One unit of enzyme is defined as the amount that liberates 1 μmol of reducing sugar/min. Protein concentration was estimated by the method of Bradford (1976), using the Bio-Rad protein assay reagent and a BSA (Boehringer Mannheim) standard.

Preparation of DNA and RNA

DNA was isolated from purified virus particles by phenol extraction as described previously (Yamada *et al.,* 1991). DNA restriction fragments separated by agarose gel electrophoresis were transferred to nylon filters (Biodyne; Pall BioSupport), hybridized with probes labeled with fluorescein (Gene Images kit; Amersham), and detected with a CDP-Star detection module (Amersham). Total RNA was isolated from uninfected and CVK2-infected *Chlorella* NC64A cells as described previously (Yamada *et al.,* 1997), blotted onto nylon filters, and hybridized with ^{32}P-labeled probes.

Washed filters were autoradiographed for 24–72 h.

Cloning and sequencing

The CVK2 chitinase gene was amplified by PCR using synthetic oligonucleotides as primers. The primers for

the 5′- and 3′-ends were 5′-ATGGCGACCGTACCAAGCACAAAAC (sense primer) corresponding to the first 25 nucleotide positions of the PBCV-1 A181R and 5′-TACGCAAATGTGTTGAATATATTTG corresponding to the last 25 nucleotide positions of the PBCV-1 A182R (antisense primer) (Lu *et al.,* 1996). The PCR product was cloned into the *Eco*RV site of pT7Blue(R) vector (Novagene), and the resulting plasmid (pvChti-1) was used for further studies. The complete chitinase gene (*vChti-1*) and its flanking regions were screened from a CVK2 genomic library by plaque hybridization as described previously (Yamada *et al.,* 1996). Subcloning was performed using M13 mp18 and mp19 vectors to make overlapping clones. Single-stranded DNA was sequenced by the dideoxy method in an Automated Laser Fluorescent (ALF) DNA sequencer (Pharmacia). The EMBL/GenBank/DDBJ databases were searched for homologous sequences, using the FASTA computer program (Pearson and Lipman, 1988). Sequence data of the CVK2 chitinase gene (*vChti-1*) and its flanking regions have been deposited with the EMBL/GenBank/DDBJ databases under accession no. AB022343.

Preparation and purification of a GST–vChti-1 fusion protein

The 2508-bp sequence containing the entire coding region of *vChti-1* was added with *Xho*I sites immediately before the translational initiation codon and immediately next to the last codon (GCG for A836) by PCR with primers of 5′-GA*CTCGAG*GATGGCGTACCAAGCACACGAC (sense primer) and 5′-T*CTCGAG*CGCAAATGTGTTGAATATATTCG (reverse primer). The amplified CVK2 DNA fragment was digested with *Xho*I and ligated to the *Xho*I site of vector pGEX-4T-3 (Pharmacia Biotech), which allowed in-frame insertion. The resulting plasmid directed the production of a hybrid protein of approximately 121 kDa composed of a 27-kDa portion of GST fused to the vChti-1 protein (94 kDa). *E. coli* BL21, deficient in the *lon* protease (Studier *et al.,* 1990) transformed with pGEX-4T-3–vChti-1, was cultured for 2–3 h at 28°C. Protein expression was then induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside for 12 h according to the manufacturer's manual. Bacterial cells were lysed by sonication in PBS, pH 7.3, containing 1% Triton X-100. The hybrid protein was purified by affinity chromatography on a glutathione–Sepharose 4B column (Pharmacia Biotech). After digestion with thrombin, the vChti-1 fragment was separated by affinity chromatography and used for the immunization of mice by standard methods.

Immunological techniques

The purified vChti-1 fragment (200 μg) was emulsified in complete Freund's adjuvant and injected subcutaneously into female mice (BALB/c, 5 weeks old). Subsequent boosts were administered at 10-day intervals with antigen in incomplete Freund's adjuvant. Mouse serum was collected 1 week after each boost. For immunoblot analysis, cellular protein samples were prepared as follows: *Chlorella* cells were harvested at various times after CVK2 infection by centrifugation at 1000 *g* for 5 min. The cells (8×10^8 cells) were resuspended in 200 μl of buffer containing 25 mM Tris-HCl (pH 7.5), 0.14 M NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 1 μg/ml pepstatin. After the addition of an equal volume of glass beads (450–500 μm; Sigma), the cell suspension was vortexed several times for 30 s on ice. Cell breakage was confirmed microscopically. After centrifugation at 14,000 *g* for 10 min at 4°C, the supernatant (60 μg protein) was electrophoresed on an SDS–10% polyacrylamide gel (Laemmli, 1970). The proteins were electroblotted onto a nylon filter (Immobilon PVDF; Millipore) with a Semi-Dry transfer cell (Bio-Rad). After blocking with 3% BSA–5% nonfat milk–0.06% Tween 20 in PBS for 4 h, the filter was treated successively with primary (anti-vChti-1) and secondary (alkaline phosphatase-conjugated sheep anti-mouse immunoglobulin G (Boehringer Mannheim) antibodies for 1 h 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.

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