JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 8893–8900 8893
DOI:10.1021/jf9016543

Characterization of an Antifungal and Cryoprotective Class I Chitinase from Table Grape Berries (*Vitis vinifera* Cv. Cardinal)

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Gene expression of a class I chitinase (*Vcchit1b*) in the skin of table grapes was analyzed as a molecular marker for changes induced at low temperature and also to study the effect of high CO_2 levels modulating transcript levels at 0 °C. An active recombinant VcCHIT1b was overexpressed in *Escherichia coli*, and as the protein was produced as insoluble inclusion bodies, it was solubilized and refolded. The purified recombinant chitinase showed an optimum pH of 6.0 and a temperature of 50 °C, retaining activity at 0 and −10 °C. Purified chitinase exerted in vitro antifungal activity against *Botrytis cinerea*. Furthermore, recombinant chitinase was able to cryoprotect lactate dehydrogenase against freeze/thaw inactivation. However, the recombinant VcCHIT1b did not show any antifreeze activity when the thermal hysteresis activity was measured using differential scanning calorimetry.

KEYWORDS: Chitinase; table grapes; carbon dioxide; antifungal activity; cryoprotective; antifreeze

INTRODUCTION

Decay caused by *Botrytis cinerea* is responsible for most of the postharvest losses of table grapes (*Vitis vinifera* L.) during storage at low temperatures; consequently, this mold is controlled mainly by extensive use of fungicides. However, fungicides and chemical treatments may cause damage to grape berries if used excessively, and some consumers develop allergic reactions. Alternative gaseous methods have also been employed to control table grape postharvest decay (*1*). In previous works, we have shown the efficacy of a 3 day pretreatment with high CO_2 levels in maintaining the quality of table grapes and controlling total decay (*2*). Likewise, although table grapes have been classified as chilling-tolerant fruits, activation of the phenylpropanoid gene expression in the first stage of grape storage at 0 °C seems to be related to the perception by the fruit of temperature shifts, which might be less noticeable in CO_2-treated grapes (*3*). On the other hand, our previous studies indicated that the efficacy of high CO_2 pretreatment in reducing total decay is not mediated by the induction of pathogenesis-related protein (PR) genes, such as chitinase and β-1,3-glucanase (*2*).

Low temperature storage is a postharvest technology used widely to extend the life of horticultural products, and in recent years, genes and metabolic pathways involved in the perception and signal transduction of plant responses to extreme temperatures have been identified (*4*). Nevertheless, most of the knowledge in this field has arisen from studies conducted on the chilling-tolerant *Arabidopsis thaliana* during cold-induced freezing acclimation. The plant genes with an expression regulated by low temperatures form a COR (cold-regulated) superfamily, many of which encode apoplastic antifreezing proteins (AFPs), late embryogenesis abundant proteins, PRs such as β-1,3-glucanases and chitinases, or other novel polypeptides (*5*).

Chitinases catalyze the hydrolysis of chitin, a linear polymer of β-1,4-linked N-acetylglucosamine residues, which is a major structural component of the cell walls of many fungi. Chitinases from several species of plants have been reported as being seven classes on the basis of primary structure, isoelectric point, and cellular localization. In plants, chitinases are induced in response to environmental stimuli, as well as being expressed in different plant tissues during normal development. Moreover, there are different works showing that chitinases, acting alone and particularly in combination with β-1,3-glucanases, contribute to plant defense against pathogen infection (*6*). In this sense, some transgenic plants overexpressing chitinase and β-1,3-glucanase genes show enhanced resistance to fungal infection in vivo (*7*). Likewise, there are several reports that have shown that chitinases isolated from monocot and dicot plants are able to inhibit the growth of some fungal pathogens in vitro. The recombinant class IV chitinase (CpCHI) from papaya showed strong antifungal activity (*8*). By contrast, a recombinant class II chitinase isolated from rice (Cht11) showed low in vitro antifungal activity, indicating that the CHT11 protein may be involved in biological processes other than the antifungal mechanism (*9*). It is interesting to note that chitinases are also expressed in certain organs or cell types of uninfected plants, suggesting that they might have important functions extending beyond their apparently limited role in plant defense. Cold-induced accumulation of chitinase transcripts has been observed in different plants, and in the case of winter rye, class I and class II chitinase gene expression was induced by low temperature, and recombinant proteins showed antifreeze activity (*10*). However, in bromegrass suspension cells,

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Published on Web 09/21/2009

a cold-responsive class I chitinase gene is unlikely to be responsible for cold-induced antifreeze activity in the culture medium by itself (*11*).

The isolation of class I chitinase genes has been reported in *V. vinifera* (*12*); even so, only limited information is presently available on its role in response to low temperature and fungal attack. The aim of the present work was to analyze the expression of a class I chitinase gene (*Vcchit1b*) in red table grapes exposed to low temperature and also to assess how high CO_2 levels modulated its transcript accumulation. Moreover, we have investigated the possible physiological role of this protein in table grapes as antifungal, cryoprotectant, and/or antifreezing by the heterologous expression of *Vcchit1b* in *Escherichia coli*.

MATERIALS AND METHODS

Plant Material. Table grapes (*V. vinifera* L. cv. Cardinal) were harvested at random in Camas (Sevilla, Spain) in July 2003. After the grapes were harvested, field-packaged bunches were transported to the laboratory, where fruits were immediately forced-air precooled for 14 h at −1 °C. After they were cooled, bunches free from physical and pathological defects were randomly divided into two lots and stored at 0 ± 0.5 °C and 95% relative humidity in two sealed neoprene containers of 1 m^3 capacity. One lot was stored under normal atmospheric conditions for 6 days (nontreated fruit), and the other was stored in a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit) for 3 days and then transferred to air for 3 days under the same conditions as the nontreated fruit. Ten clusters were sampled periodically, berries from five clusters (approximately 300 g each cluster) were peeled, and the skin was frozen in liquid nitrogen, ground to a fine powder, and stored at −80 °C until analysis.

RNA Gel Blot Hybridization. Samples of denatured total RNA (10 μg) extracted from the skin of grapes were fractionated and blotted as described in Sanchez-Ballesta et al. (*3*). Equal loading was confirmed by membrane staining with methylene blue. The *Vcchit1b* DNA probe was random-primer labeled with α^{32}P-dCTP. Filters were prehybridized and hybridized at 65 °C in 7% sodium dodecyl sulfate (SDS), 0.33 M phosphate buffer, pH 7.2, and 1 mM ethylenediaminetetraacetic acid (EDTA), then washed twice in 2× SSC and 0.1% SDS at room temperature and twice in 0.1× SSC and 0.1% SDS at 65 °C, and exposed to Kodak X-Omat SX film at −80 °C. Autoradiographs were digitally scanned, and band densities were quantified by image densitometry using Scion Image software (Scion Corp., Frederick, MD). A value of 100% was assigned to the maximum optical density value achieved in each Northern blot, and the rest of the optical densities were normalized to the maximum value and expressed as percentages of relative accumulation (RA).

Production of the Recombinant VcCHIT1b Protein in *E. coli*. The cDNA fragment of *Vcchit1b* (GenBank accession number DQ267094) without the N-signal peptide was amplified using forward primer (5′- ATTATCGGATCCGAGCAATGTGGAGGGCAAGCT-3′) and reverse primer (5′-GGC GAATTCTTAGATGGTGTCCAGCAGGAG-3′) containing *Bam*HI and *Eco*RI restriction sites, respectively. The polymerase chain reaction (PCR) conditions were 30 cycles of 40 s at 95 °C, 40 s at 53 °C, and 40 s at 72 °C. The amplified DNA fragments were digested with *Bam*HI and *Eco*RI and ligated into the multicloning site of the pTrcHisA (Invitrogen, Carlstad, United States) previously digested with the same enzymes. The vector pTrcHisA-VcCHIT1b was transformed into *E. coli* strain TOP10 cells, which were grown at 37 °C in LB medium with 50 μg mL^{-1} ampicillin and 0.8 mM glucose until the optical density at 600 nm reached 0.6 and then induced with 1 mM isopropyl-ss-D-thiogalactoside (IPTG). After 3 h of induction at 37 °C, cells were harvested by centrifugation at 1760*g* for 20 min at 4 °C and frozen. Cells were resuspended in lysis buffer (50 mM sodium Tris-HCl, pH 7.8, and 1 mM EDTA) and disrupted with 1 g of glass beads (150−252 μm, Sigma) in a FastPrep machine (FP120, Bio101, Savant). The cell extract was centrifuged at 13500*g* for 10 min at 4 °C to remove the unbroken cells and the supernatant. The pellet, containing the inclusion bodies and the glass beads, was washed in three volumes of wash buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 M urea), kept at room temperature for 5 min, and centrifuged as above. The protein was renatured from inclusion bodies using the method described by Kirubakaran and Sakthivel (*13*). First, the buffer was removed by resuspending and washing the pellet using sterile distilled water five times and centrifuged as above. To renature the protein, the pellet was dissolved in a 10× volume of 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 2.5 mM β-mercaptoethanol, and 0.1% (w/v) Tween 20 (denaturation−renaturation buffer; DR buffer) containing 8 M urea and incubated at room temperature for 1 h. The mixture was centrifuged at 10000*g* for 20 min at 4 °C. The supernatant was dialyzed gradually against DR buffer containing progressively low concentrations of urea (6, 4, 3, 2, 1, and 0 mM) during 18 h and finally using sterile distilled water for 24 h. Several aliquots of chitinase-dialyzed extract were lyophilized to obtain a concentrated chitinase powder for the antifungal activity. A negative control was obtained after expression and purification of the empty vector pTrcHisA into *E. coli* strain TOP10 cells according to procedure described above. The protein concentration was determined by the Bradford method (*14*) using a protein-dye reagent (Bio-Rad) and bovine serum albumin (BSA, Sigma) as a standard.

Gel Electrophoresis and Protein Blot Analyses. Protein analysis was performed on 12% SDS−polyacrylamide gel electrophoresis (PAGE) using Mini-Protean II Cell (Bio-Rad) equipment. Protein samples were reduced by boiling for 10 min in 2× SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.05% bromophenol blue). Protein bands were visualized by staining with Coomasie brilliant blue R-250. The molecular mass of the separated polypeptides was estimated by comparing it with the mobility of prestained standard low molecular mass range proteins (Bio-Rad).

After a standard PAGE was performed, proteins were electrotransferred to PVDF membranes (GE Healthcare) with a Mini Trans-Blot Cell (Bio-Rad). The Western blot was probed with a 1:5000 dilution of polyclonal anti-PR-Q sera from tobacco, kindly provided by Dr. Fritig (Strasbourg, France), and was detected with a 1:5000 dilution of rabbit antiserum against IgG horseradish peroxidase conjugate (GE Healthcare). The immunocomplexes were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare).

Chitinase Activity from Grape Skin Extracts and Recombinant Protein. Protein was extracted by homogenizing ground frozen berry skin tissues (250 mg fresh weight) at 4 °C in 5 mL of 100 mM sodium acetate buffer, pH 5.0, and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 27000*g* for 30 min, and the clarified supernatant was recovered. All steps were carried out at 4 °C. The chitinase activity from protein extracts, and recombinant chitinase was assayed using a commercial blue enzyme substrate, CM-chitin-RBV solution (Loewe), based on the precipitability of a nondegraded, highly polymerized substrate when acid is added, as described by Merodio et al. (*15*). The reaction was carried out for 30 min at 37 °C. One unit of chitinase activity (U) was defined as the increase in absorbance at 550 nm per mL per minute (ΔA_{550} mL^{-1} min^{-1}). The specific enzyme activity was expressed as U mg^{-1} of protein.

Enzyme Characterization. The effect of pH and temperature on activity of the purified chitinase was determined by measuring the specific activity of 0.1 mg of purified VcCHIT1b under standard assay conditions. The optimum pH value was determined over the pH range 4.0−9.0 in 100 mM suitable buffer (sodium acetate, pH 4.0−6.0; sodium phosphate dibasic, pH 7.0; and Tris-HCl, pH 9.0). The temperature optimum was examined by incubating standard assay mixtures at temperatures ranging between −10 and 70 °C in 100 mM sodium acetate buffer, pH 6.0. The enzymatic assays carried out at 0 and −10 °C were done in refrigerated chambers. In this sense, under subzero temperatures, the reaction components exist like a supercooled solution throughout reaction time. The activation energy (E_a) of the enzymatic reaction was calculated by measuring the slope of the linear form of the Arrhenius plot, which was made based on the reaction rates (in U mg^{-1}) measured at given temperatures:

$$\log V = \frac{-E_a}{2.3R} \frac{1}{T} + \log A$$

in which R is the molar gas constant, T is the absolute temperature, and V is the specific reaction rate constant.

Assay of Protein Antifungal Activity. Recombinant purified VcCHIT1b was tested for antifungal activity toward *B. cinerea* (strain provided by the Spanish Type Culture Collection, Universidad de Valencia,

Spain) by the hyphal extension-inhibition bioassay. All manipulations were carried out under sterile conditions. Fungi were grown at 25 °C on potato dextrose agar (PDA) for 7 days, after which time spores were harvested from sporulating colonies and suspended in sterile 1% (v/v) Tween 20 in water. The concentration of spores in suspension was adjusted to 1.5 × 10^6 spores mL^{-1}. The inhibition assay of hyphal elongation was carried out in 100 mm × 15 mm Petri plates containing 20 mL of PDA. Freshly prepared inoculum (15 μL) was placed in the center and incubated for 24 h. After the mycelial colony had developed, sterile blank paper disks (0.7 cm in diameter) were placed around it at a distance of 1 cm. The disks assayed contained 20 mM sodium acetate buffer (pH 5.0) as a negative control and an aliquot containing 25 μg of purified VcCHIT1b along with an aliquot containing 100 μg of bacterial chitinase (*Streptomyces griseus*, Sigma) as a positive control. Both proteins were dissolved in 20 mM sodium acetate buffer (pH 5.0). The plates were incubated in the dark for 35 h at 25 °C and for 12 days at 0 °C until mycelial growth had enveloped the positive control disk and had formed zones of inhibition around disks containing samples with antifungal activity. Growth inhibition zones were measured according to Iseli et al. (*16*).

Assay for Cryoprotective Activity. The in vitro cryoprotective activity was determined following the method described by Lin and Thomashow (*17*), with slight modifications. Lactate dehydrogenase (LDH, EC 1.1.1.23, type V-S from rabbit muscle, Sigma) was diluted to 87.6 μg mL^{-1} in 20 mM potassium phosphate buffer, pH 7.5, and used as a freeze-labile stock enzyme solution. Samples of either VcCHIT1b, empty vector pTrcHisA purified protein fractions, or BSA (dissolved in sterile distilled water) were diluted to varying concentrations and mixed with 2.364 μg of LDH from the stock solution. The resulting solution (300 μL) was frozen in liquid nitrogen for 30 s and then thawed at room temperature for 5 min. The freeze−thaw process was carried out three times, and the residual LDH activity was measured. The LDH enzymatic activity was determined in aliquots containing 30 μL of the mixtures in a final volume of 1.5 mL of the reaction assay buffer (80 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM pyruvate, and 30 mM NADH) at room temperature. NAD^+ production was monitored as the decrease of absorbance at 340 nm for 4 min. The data are presented as the percentage of the activity present in unfrozen controls.

Antifreeze Activity Measurement. Differential scanning calorimetry (DSC) was used to determine antifreeze activity, measuring the thermal hysteresis activity (THA) by using the method described by Romero et al. (*18*). An antifreeze protein (type III) from Antarctic fish, kindly provided by Dr. DeVries (IL), and BSA (AFP-free solution) were used as standards to make a comparison between solutions with and without antifreeze activity. Recombinant VcCHIT1b, AFP-III, and BSA proteins (dissolved in sterile distilled water) were used in a concentration of 5 mg mL^{-1}. THA was defined as the difference between the hold temperature (T_h) and the recrystallization temperature (T_c).

Statistical Analysis. The data from at least three replicates per sample were subjected to analysis of variance (one-way ANOVA) using the LHD test to determine the level of significance at $P \leq 0.05$ (Statgraphics Plus version 5.1, STSC, Rockville, MD).

RESULTS

In previous work, the full length of the *Vcchit1b* cDNA was isolated from the skin of table grapes (*2*). The predicted amino acids of VcCHIT1b shared homology with class I chitinases containing a putative signal peptide of 20 amino acids followed by a cysteine-rich chitin binding domain, founded in the N-terminal sequence. Sequence comparison with chitinases with known structure reveals the presence of three disulfide bonds, which are often conserved in these plant proteins and also the active-site glutamic acids corresponding to residues Glu130 and Glu152 (**Figure 1**).

To study the mechanisms associated with table grape response at 0 °C and to determine whether high CO_2 levels could modulate them, we have analyzed changes in *Vcchit1b* mRNA accumulation and chitinase activity of protein extracts during the first stage of storage (**Figure 2**). Northern experiments revealed differences between the accumulation of *Vcchit1b* in nontreated and CO_2-treated grapes. Low temperature sharply increased the levels of the transcript after 3 and 6 days of storage in grape skins stored in air. However, the rise in the accumulation of *Vcchit1b* mRNA in the CO_2-treated grapes was lower after 3 days at 0 °C, and it was not maintained when treated grapes were transferred to air for 3 days at 0 °C (**Figure 2A**). The chitinase activity increased significantly after 3 days at 0 °C in nontreated fruit, maintaining the levels after 6 days. By contrast, in CO_2-treated grapes, the chitinase activity only increased when fruit where transferred to air (**Figure 2B**).

To determine whether the isolated *Vcchit1b* cDNA encoded a protein with in vitro chitinase activity and to analyze its functionality, the cDNA fragment corresponding to the mature protein without the N-terminal signal peptide was expressed in TOP10 *E. coli* cells as a pTrcHisA-VcCHIT1b fusion protein. Bacteria carrying the recombinant vector were grown at 30 °C in LB medium with ampicillin (50 μg mL^{-1}) and 0.8 mM glucose, and the VcCHIT1b protein was induced in the presence of 1 mM IPTG at 37 °C for 3 h. Cell extracts were centrifuged, and both the pellet and the supernatant were analyzed by Western blot with an antiserum against tobacco PR-Q (**Figure 3A**). Results indicated that the recombinant protein remained in the IPTG-induced pellet fraction, indicating that the major part of the protein was produced as insoluble inclusion bodies. The noninduced control did not express chitinase protein. Despite the variations in the assayed conditions such as the growth temperature, the IPTG concentration, and the induction time, we were not able to produce soluble protein (data not shown). VcCHIT1b was solubilized and refolded from inclusion bodies by using the Kirubakaran and Sakthivel method (*13*), and the purified recombinant protein showed a single protein band around 41 kDa in SDS-PAGE (**Figure 3B**). Likewise, to confirm the identity of the purified protein, Western blot analysis was carried out, and a positive band with the same molecular mass was observed (**Figure 3C**). The yield of purified chitinases was 8.75 mg L^{-1} with a chitinase activity of 5.36 U mg^{-1} as determined by using colloidal CM-chitin-RBV substrate (**Table 1**). These results indicated that VcCHIT1b produced in *E. coli* cells was correctly folded into an active enzyme.

The effects of pH and temperature on the hydrolytic activity of VcCHIT1b against this colloidal substrate were measured with a pH ranging from 4.0 to 9.0 and temperatures from −10 to 70 °C. The maximum activity was observed at pH 6.0 and 50 °C (**Table 1**). Although about 50% of the maximal activity remained within the pH 4.5−8.0 range, the purified enzyme lost over 80% of its activity, decreasing sharply below pH 4.0 and above pH 8.5. Interestingly, the recombinant protein displayed 50% of the relative activity over a high range of temperatures (0−50 °C) and at −10 °C showed 20% of the maximum activity observed (data not shown). To clarify the observed thermodependence of the catalytic behavior of the recombinant chitinase, we determined its energy of activation (E_a) based on linear regression of the Arrhenius plot for the temperature range from −10 to 50 °C. The calculated E_a of the recombinant VcCHIT1b was 7.12 kJ mol^{-1} (**Table 1**).

We analyzed the antifungal activity of the recombinant VcCHIT1b at 25 and 0 °C using the hyphal growth inhibition assay on agar plates with *B. cinerea* as the test fungus. The assays carried out using a concentration of 25 μg (0.13 U) of purified recombinant protein showed that VcCHIT1b is an effective antifungal agent against this phytopathogenic strain, measuring an inhibition zone of 0.45 cm when plates were incubated at 25 °C and 0.31 cm at 0 °C. The commercial endochitinase from *S. griseus* used as a positive control had a lower antifungal activity than that observed for VcCHIT1b with an inhibition

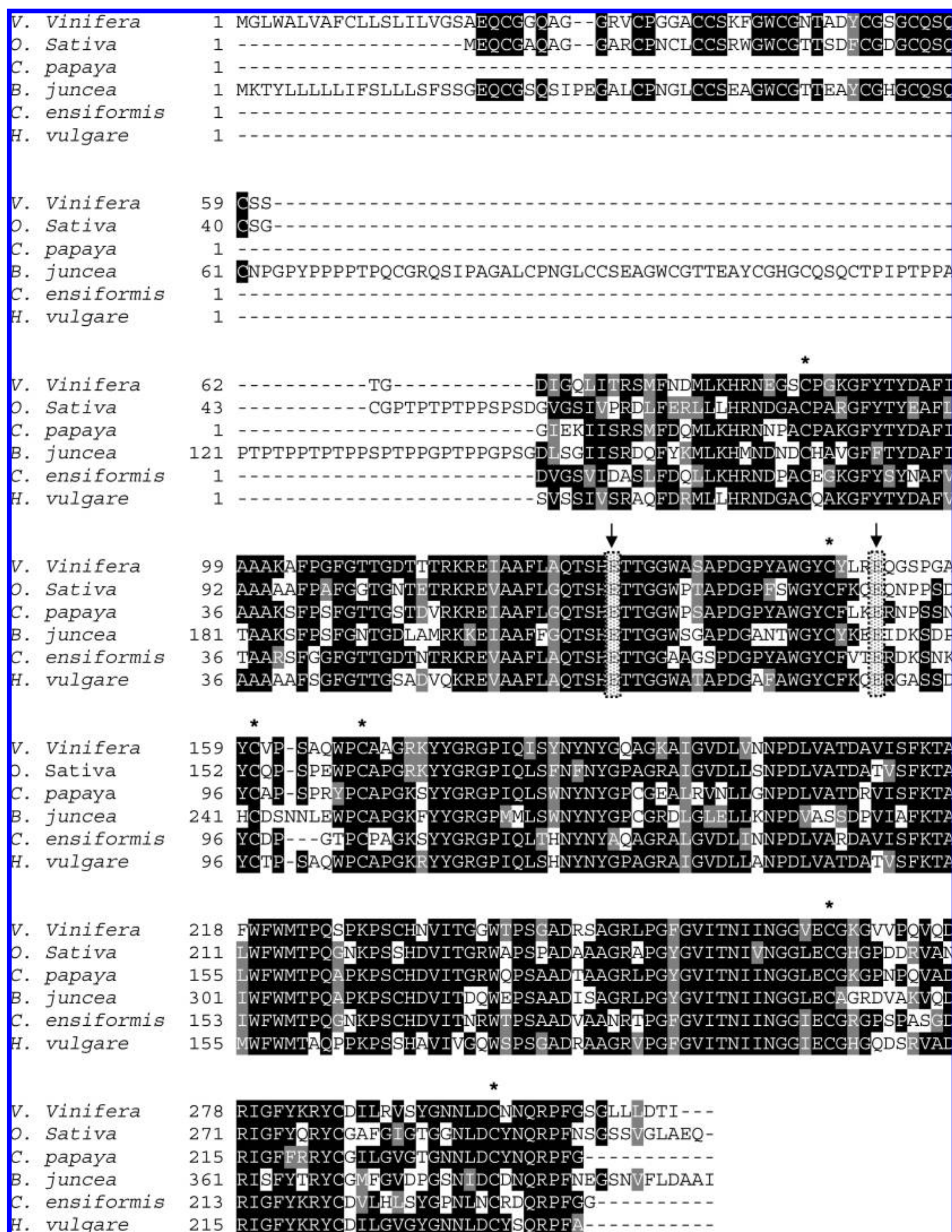

Figure 1. Alignment of the deduced amino acid sequence of chitinase from *V. vinifera* cv. Cardinal (accession number DQ267094) and plant chitinases with known structures from Oryza (pdb code 2dkv_A), *Carica papaya* (pdb code 3cgl_A), *Brassica juncea* (pdb code 2z38_A), *Canavalia ensiformis* (pdb code 1dxj_A), and *Hordeum vulgare* (pdb code 2baa_A). Identical amino acids and conservative substitutions are shown on a black or gray background, respectively. The active-site glutamic acids are boxed and indicated by an arrow, and cysteine residues involved in disulfide bonds are indicated by asterisks.

zone of 0.28 cm at 25 °C and 0.13 cm at 0 °C (**Figure 4**). Incubation of plates at low temperatures showed both reduction of growth rate and inhibition zone of *B. cinerea*. However, bacterial endochitinase exhibited a higher relative decrease of inhibition zone than VcCHIT1b (2.15 vs 1.45 times, respectively).

To further analyze the in vitro functionality of recombinant protein, we investigated the ability of the recombinant chitinase to preserve LDH activity following freeze−thaw cycles as compared with the level of cryoprotection provided by BSA. Without the addition of a cryoprotectant, three freeze−thaw cycles resulted in more than an 80% reduction of LDH activity (**Figure 5**). The addition of VcCHIT1b or BSA, in protein:enzyme molar ratios from 0.5 to 8, significantly protected LDH activity, but recombinant VcCHIT1b was more efficient than BSA in protecting LDH. At a molar ratio of 2, VcCHIT1b maintained 100% of initial LDH activity. We also determined the concentration of protein that is able to preserve 50% LDH activity after freeze−thaw cycles (PD50). The recombinant chitinase showed a PD50 of 3.02 μg mL^{-1}, which expressed on a molar basis is 96.06 nM. In comparison, the addition of 14.15 μg mL^{-1} (214.39 nM) of BSA

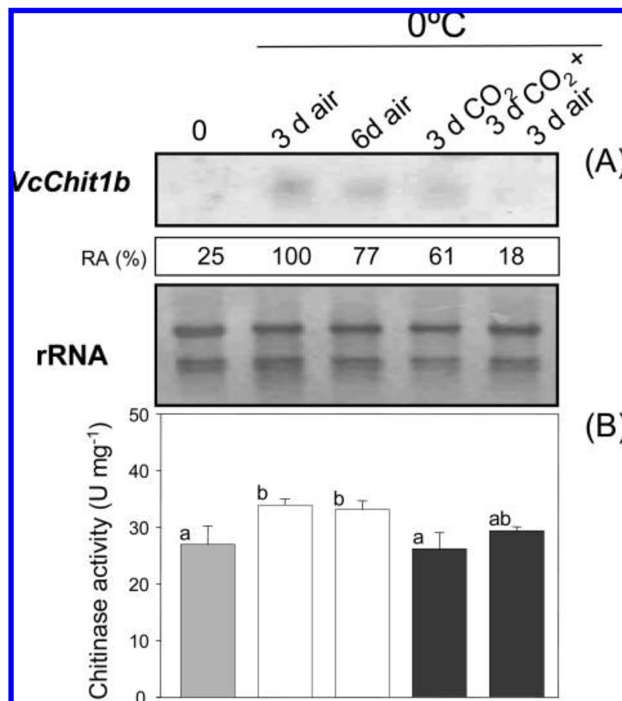

Figure 2. Effect of low temperature and high CO_2 levels on *Vcchit1b* mRNA accumulation (**A**) and chitinase activity (**B**) in the skin of Cardinal table grapes. (**A**) Ten micrograms of total RNA from the skin was fractionated by gel electrophoresis, blotted, and hybridized with the full-length *Vcchit1b* probe. The intensity of the bands was quantified by scanning densitometry of the autoradiographs. Optical density values were normalized to the maximum value and expressed as percentages of RA. The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining. (**B**) Time courses of chitinase activity in the skin of nontreated and CO_2-treated Cardinal table grapes at 0 °C. Error bars represent SE ($n = 9$). Values labeled with the sale letter are not different at the 5% significance level.

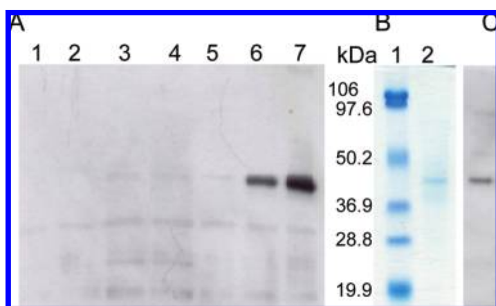

Figure 3. SDS-PAGE analysis of recombinant VcCHIT1b produced in *E. coli*: (**A**) Western blotting detected by an anti-PR-Q sera from tobacco. Lanes: 1, total cell proteins from noninduced *E. coli* TOP10 containing pTrcHisA-VcCHIT1b; 2−4, supernatant from centrifugation of 0, 2, and 3 h induced cells; and 5−7, pellet from centrifugation of 0, 2, and 3 h induced cells. (**B**) Lanes: 1, protein molecular weight marker; and 2, purified chitinase after solubilization and refolding from inclusion bodies. (**C**) Western blotting of purified chitinase after incubation with PR-Q antiserum from tobacco.

was required to obtain the same results. On the other hand, we determined the cryoprotective activity of purified fraction of empty vector pTrcHisA. After an initial small decrease, this negative control showed a relatively constant LDH activity of about 15% (**Figure 5**).

Table 1. Biochemical Properties of Recombinant VcCHIT1b

	VcCHIT1b
specific activity (U mg^{-1})	5.36
pH optimum	6.0
temperature optimum (°C)	50.0
pH range for 50% activity	4.5−8.0
T^a range for 50% activity	0−50.0
E_a (kJ mol^{-1})	7.12

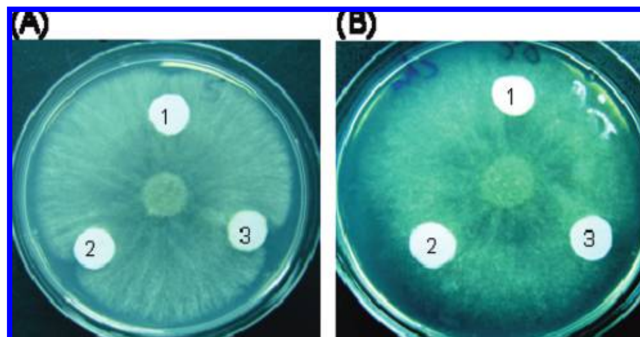

Figure 4. Inhibition of hyphal elongation of *B. cinerea* by recombinant VcCHIT1b at 25 (**A**) and 0 °C (**B**). (1) Control, 30 μL of 20 mM sodium acetate buffer (pH 5.0), (2) 100 μg of bacterial chitinase *S. griseus* in 30 μL of 20 mM sodium acetate buffer (pH 5.0), and (3) 25 μg of recombinant chitinase in 30 μL of 20 mM sodium acetate buffer (pH 5.0).

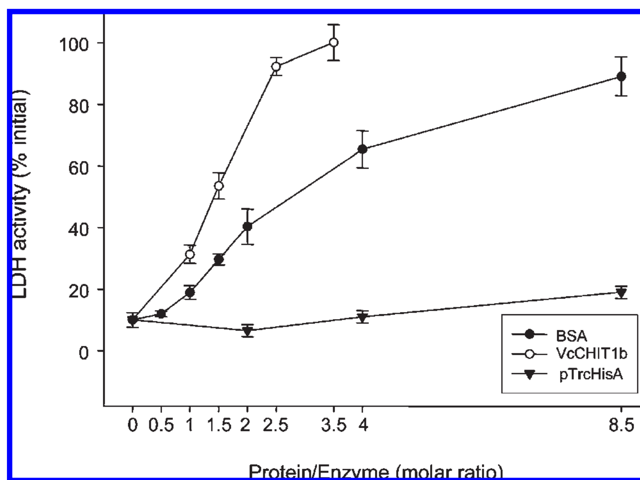

Figure 5. Cryoprotection of LDH by recombinant VcCHIT1b. (**A**) The LDH solution was frozen with different concentrations of VcCHIT1b, BSA, and a purified fraction of empty vector pTrcHisA. The samples were thawed at room temperature, and the LDH activity was measured. The relative activity represents the amount of LDH activity remaining after a freeze−thaw treatment as a percentage of the control enzyme activity. Error bars represent SE ($n = 3$).

The in vitro antifreezing activity of recombinant VcCHIT1b was studied by measuring the THA by DSC. In a positive control (AFP-III protein solution), a delay of 0.4 °C in the onset temperature of refreezing was observed. In recombinant VcCHIT1b protein and negative control (BSA), the exothermic peak appeared without this delay. Therefore, the chitinase showed a slow recrystallization of the melted part just after the temperature had dropped (**Figure 6**). This indicates that the VcCHIT1b had no thermal hysteresis effect, and consequently, it had not exhibited the ability to modify the normal growth of ice crystal.

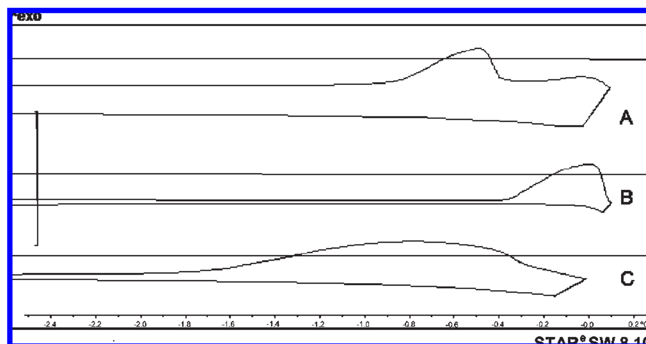

Figure 6. Refreezing DSC curves (0.5 °C min^{-1}) of partially melted protein−water systems. (**A**) AFP-III, (**B**) BSA, and (**C**) recombinant VcCHIT1b. The hold temperatures (T_h) of the curves from **A** to **C** are 0.1, 0.1, and 0.0 °C, respectively. The vertical line represents a normalized heat flow of 20 mW.

DISCUSSION

Table grapes are not susceptible to injury at low (not freezing) temperatures, but previous studies have indicated that the fruits of Cardinal cultivar are sensitive to temperature shifts at 0 °C, activating class I β-1,3-glucanase and phenylpropanoid gene expression. These changes were reduced however, in the case of fruit that had undergone the 3 days high CO_2-treatment (*3*, *18*). To understand the mechanisms implicated in the response of table grapes to low temperature and the role played by high CO_2 in modulating these responses, we describe in this report the characterization of a cold-responsive transcript, *Vcchit1b*, encoding a chitinase.

Chitinases belong to a large gene family that has been subdivided into five major classes based on amino acid sequences derived primarily from sequence analyses of cDNA clones (*6*). In previous work, we isolated a full-length chitinase (*Vcchit1b*) cDNA from the skin of table grapes (*2*). The predicted amino acid sequence of VcCHIT1b contains typical domains of class I chitinases such as a hydrophobic signal peptide of 20 amino acids followed by a cysteine-rich chitin binding domain in the N-terminal sequence. Sequence comparison with chitinases of class I and II with a known X-ray structure (**Figure 1**) revealed that Glu130 and Glu152 are well conserved and presumably involved in hydrolytic catalysis. Glu130 is believed to act as the proton donor, and Glu152 has been proposed as having a dual role, promoting nucleophilic attack by water on the anomeric carbon of the sugar and stabilizing the charged intermediate (*19*−*23*). Likewise, the presence of four disulfide bonds, which had been predicted by using ScanProsite analysis and sequence alignment, was found to be well-conserved in the different chitinases classes.

Different studies have shown that plant chitinases have different roles such as storage proteins (*24*), PR activity (*6*), as well as properties typical of antifreeze proteins (*10*, *25*). In previous work, we observed that the increase in the levels of *Vcchit1b* mRNA was paralleled by the change in total decay at the end of storage at 0 °C (*2*). In this work, we have studied the effect of low temperature and high CO_2 levels on the accumulation of class I chitinase transcript and chitinase activity during the first stage of storage before the appearance of natural decay in table grapes (**Figure 2**). Low temperature increased the accumulation of *Vcchit1b* mRNA and chitinase activity after 3 days, decreasing slightly after 6 days the levels of the mRNA but not the chitinase activity. In accordance with our findings, chitinase gene expression induction has been reported during cold acclimation. In winter rye, class I (*CHT9*) and class II (*CHT46*) Chitinase mRNAs accumulated after 5 weeks of cold acclimation but degraded rapidly when the plants were shifted to a warm temperature (*10*). Furthermore, expression of a class I chitinase (*BiCHT1*) mRNA was increased by low temperature in bromegrass suspension cells, suggesting that the product may be involved in cold acclimation (*11*). High CO_2 levels reduced the accumulation of the *Vcchit1b* transcript and chitinase activity observed at 0 °C, reaching values lower than those observed after 6 days in nontreated grapes. Likewise, when treated fruits were transferred to air for 3 days, the accumulation of the transcript decreased, increasing the chitinase activity (**Figure 2**). Bertrand et al. (*26*) studied the expression of a class III chitinase in cold-tolerant alfalfa grown and acclimated under elevated atmospheric CO_2 conditions and found that transcripts levels were lower in cold-acclimated than in nonacclimated roots. Our results reinforce the idea that high levels of CO_2 applied at 0 °C controlled the responses induced in nontreated grapes in the first stage of exposure to 0 °C.

Although characterization of chitinase proteins has been reported, little is known about their function in fruit-producing crops. In particular, only one class III chitinase has been characterized in *V. vinifera* (*27*). Despite the fact that recombinant proteins produced in bacteria are often formed as insoluble, functional proteins can still be recovered by in vitro preparative refolding from inclusion bodies. In fact, expression of *Vcchit1b* cDNA in *E. coli* was difficult because of this; however, after solubilization and refolding processes, the recombinant VcCHIT1b showed a single protein band in SDS-PAGE (**Figure 3**) with a molecular mass around 41 kDa, a value that agreed with the molecular mass calculated from deduced polypeptide. The purified VcCHIT1b displayed chitinase activity with a maximum at pH 6.0 and 50 °C, and it also maintained about 50% of its maximal activity over a broad range of pH values (4.5−8.0) and temperatures (0−50 °C) (**Table 1**). Different studies have also shown that recombinant plant chitinases displayed activity over a wide range of pH values and temperature intervals, such as pH 3.0−6.0 and 20−50 °C for *V. vinifera* class III chitinase (*27*), pH 5.0−9.0 and 10−50 °C for papaya class III chitinase (*8*), and pH 4.0−7.0 and 20−50 °C for rice class II chitinase (*9*). However, it is important to note that recombinant VcCHIT1b displayed 50 and 20% of relative activity at 0 and −10 °C, respectively. Similarly, the E_a for VcCHIT1b was much lower than values obtained for other plant chitinases (*28*). In previous work, we observed that a recombinant β-1,3-glucanase from table grape showed 27% of relative activity at 0 °C and a low E_a (*18*). Moreover, in winter rye, recombinant β-1,3-glucanase retained 65% of its maximal activity at 0 °C but reduced by 86% at −4 °C (*29*). Nevertheless, to our knowledge, this is the first report where a plant chitinase showed low values of E_a and chitinase activity at subzero temperature. These thermal properties are similar to those displayed by cold-active enzymes (*30*). These enzymes are believed to need a high degree of structural flexibility to retain their activities at low temperature. It is thought that the flexible structures of these enzymes consist mainly of a decreased number and strength of intramolecular interactions. In this sense, the high content of cysteines in the class I chitinases (with a potential for forming several intramolecular disulfide bridges and a structurally stable molecule) and its high catalytic activity at low temperature seem like a paradox. However, similar results have been reported for a cold-adapted lysozyme, which also revealed a particularly high number of cysteines and mild thermal stability (*31*).

The antifungal potential of plant chitinases by lysing fungal tips and inhibiting growth is well-known, and in this study, we have shown that the in vitro antifungal activity of VcCHIT1b

inhibits *B. cinerea* growth. (**Figure 4**). Similar results were obtained when a recombinant class III chitinase from Koshu grape leaves was assayed at 25 °C (*27*). Furthermore, transgenic grapevine plants expressing a rice class I chitinase have been shown to have enhanced resistance against powdery mildew caused by *Uncinula necator* (*32*). Although the inhibition was higher at 25 °C, the relative decrease of inhibition zone was significantly lower than the mesophilic bacterial endochitinase, suggesting that its psychrophilic character has been preserved in an antifungal assay. These results indicated that one potential role of this PR in the defense mechanisms of *V. vinifera* is related to a direct attack to pathogenic fungi. Taking into account that *B. cinerea* is a major cause of postharvest rot of perishable plant products and that it is also able to infect at low temperatures, our recombinant cold-active chitinase could conceivably be used as a biofungicide to prolong the cold storage period of fruit or economic crops due to its combination of low temperature activity and ability to inhibit in vitro growth of this necrotrophic pathogen.

Other functions described in some PRs are their potential role as cryoprotectants and/or growth inhibitors of ice within the context of cold protection. Hincha et al. (*33*) reported a tobacco β-1,3-glucanase with cryoprotective activity maintaining thylakoid membrane integrity. Likewise, a thaumatin-like protein from groundnut manifested in vitro cryoprotective activity by preventing freeze-induced denaturation of LDH, which was 2.5 times higher than BSA (*34*). In winter rye, the products of a class I and class II chitinase cDNAs exhibit antifreeze activity when they accumulate in *E. coli* (*10*). Even in previous work, we have partially purified a basic chitinase from cherimoya protein extracts, which was induced in response to high CO_2 levels and showed cryoprotective activity 2.7 times higher than BSA (*35*). However, this does not appear to be a general characteristic of stress-induced PR proteins. A tobacco class I chitinase (*33*) and a citrus class III β-1,3-glucanase (*36*) did not show any cryoprotective activity. Moreover, class I endochitinase purified from cold-acclimated winter rye exhibited ice-binding activity, whereas a class I endochitinase isolated from pathogen-infected tobacco did not show any antifreeze activity (*25*). Furthermore, a class I chitinase from bromegrass expressed in *E. coli* did not show any antifreeze activity by affecting the growth of ice crystals (*11*). In this study, we have demonstrated the in vitro cryoprotective activity of VcCHIT1b against LDH (**Figure 4**) but not the antifreeze activity when the THA was determined by DSC (**Figure 5**). With regard to BSA, the cryoprotective activity of the recombinant protein was 2.2 times higher on a molar basis. It is important to emphasize that when comparing BSA, similar or more conclusive results than ours have been observed with different cold-induced dehydrins (*37*), although this is the first report showing this activity in a class I chitinase. The mechanism by which dehydrins cryoprotect LDH is still unknown. Ukaji et al. (*38*) indicated that the lower cryoprotective activity of a PR-10 from mulberry with respect to other cryoprotectants could be partially related to the lower ratio of polar residues (40%) on the surface of the protein. The percentage of dehydrin protein polar residues was around 50% (*39*), as was observed in VcCHIT1b. However, we have identified a class I β-1,3-glucanase with 40% of polar residues that displayed a cryoprotection similar to BSA (*18*). On the other hand, there is no evidence for the antifreeze activity of our recombinant chitinase. The gentle slope of VcCHIT1b thermogram is not due to a slow specially inhibition ice recrystallization but rather to be a known general effect of the presence of large protein molecules in ice (*40*). The biological significance of this property may simply be an epiphenomenon brought about as a consequence of its structure. As a result of its high hydrophilicity and concomitant ability to bind water, the grape chitinase may bind to ice surfaces and significantly slow grain boundary migration. In this sense, we have calculated the percentage of hydrophobic residues of BSA and VcCHIT1b using the GRAVY parameter of ProtParam Tool (ExPASy server). The BSA value was higher than that of chitinase (-0.136 vs -0.433), providing an explanation to different recrystallization kinetics of BSA and VcCHIT1b.

In short, an active cold chitinase from table grapes was overexpressed in *E. coli*, purified, and characterized, showing activity over a wide range of pH and temperatures and even retaining this activity at subzero temperature. The plant recombinant chitinase described in this work differs from those previously reported in the following aspects: in vitro cryoprotective activity and retaining catalytic activity at subzero temperature. Both of these differences make VcCHIT1b a very interesting candidate for a number of biotechnological applications in which the combined use of a defense enzyme and a cryoprotectant is required to provide ample protection for plant cells during storage at low temperature. Another remarkable aspect of this study is the fact that low temperature induced defense responses involved in cold adaptation and fungal attack, which were reduced by high CO_2 levels.

ACKNOWLEDGMENT

We are grateful to B. Fritig (Strasbourg, France) for his generosity in providing PR-Q antisera, P. Lopez Garcia for her suggestions about solubilization and refolding of inclusion bodies, and Frutaria SAT for providing table grapes.

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Received May 18, 2009. Revised manuscript received September 2, 2009. Accepted September 02, 2009. This work was supported by grants from the Spanish Ministry of Education and Science (AGL2008-02949/ALI) and Comunidad Autonoma de Madrid (200670M050). I.R. was supported by a postgraduate I3P contract from the CSIC, and M.T.S.-B. was supported by a Ramon y Cajal contract from MEC, Spain. C.F.-C. and O.G. were supported by a predoctoral fellowship from MEC, Spain.