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Full Length Research Paper

Cloning and functional characterization of a class III chitinase gene from grapevine: Inhibition of fungal growth by recombinant VvChiF III

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To characterize the structure and function of chitinase genes, a class III chitinase gene (*VvChiF* III) was isolated from *Vitis vinifera* cv. Flame seedless. The *VvChiF* III open reading frame comprised 894 nucleotides with no introns and encoded a protein of 297 amino acids. The amino acid sequence encoded by *VvChiF* III showed a high identity to that of a class III chitinase isolated from *V. vinifera* cv. Koshu and to other acidic chitinase. Analysis of the VvChiF III amino acid sequence showed that this gene corresponds to the Glyco-hydro-18 super family that consisting of a signal peptide with the length of 25 amino acids. Purified VvChiF III showed chitinase activity toward the soluble substrate, glycolchitin and antifungal activity against *Botrytis cinerea*.

Key words: *Vitis*, chitinase genes, recombinant protein, antifungal activity, SNPs.

INTRODUCTION

Through continuous exposure to a wide range of pathogens, plants have evolved a range of mechanisms to resist pathogen invasion (Allen et al., 2004). The first line of defense to pathogen attack are the cuticle and the cell wall which represent physical barriers to penetration. Secondly plant cells contain both preformed and inducible antimicrobial compounds such as phenols, sulphur compounds, phenolic glycosides, unsaturated lactones, saponins, cyangenic glycosides, glucosinolates, phytoalexins together with pathogenesis related (PR) proteins (Osbourn 1996; Dixon, 2001; Van Loon et al., 2006). In recent years, several molecular approaches have been used to investigate resistance against fungal diseases in grapevine that have provided some insight into these defence mechanisms.

Two groups of PR proteins that have a significant role in the defence against invading fungal pathogens are the β-1,3-glucanase (PR group 2) and chitinases (PR group 3) and plant chitinases are thought have an important role in the plant defence system against fungal pathogens due to its ability to hydrolyze chitin which is abundant in the cell walls of many fungi. Chitinase induction during fungal attack as well as *in vitro* inhibition of fungal growth has been demonstrated (Schlumbaum et al., 1986; Linthorst, 1991; Arlorio et al., 1992; Collinge et al., 1993; Herrera-Estrella and Chet, 1999; Ano et al., 2003). Antifungal activity has also been demonstrated for PR proteins in groups 1 (Alexander et al., 1993; Niderman et al., 1993), 4 (Ponstein et al., 1994), and 5 (Vigers et al., 1991, 1992; Woloshuk et al., 1991). Enhanced resistance to fungal pathogens has also been demonstrated in transgenic plants over-expressing chitinase or β-1,3-glucanase, with a synergistic benefit where both genes are present (Broglie et al., 1991; Jach et al., 1995; Jongedijk et al., 1995; Grison et al., 1996; Asao et al., 1997; Yamamoto et al., 2000). Thus, several lines of evidence are consistent with the hypothesis that chitinases are an important component of plant defense systems. Consequently, plant chitinases are the subject of intensive research that may ultimately lead to disease resistant crops and decreased use of ecologically harmful

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pesticides. This area of research is also of particular interest in grapes because of their widespread production for winemaking, dried fruit, and table grapes and their susceptibility to a wide range of fungal pathogens such as powdery mildew, downy mildew and botrytis.

Although the genes encoding PR proteins do not show high levels of constitutive expression in plants, however, are induced in response to pathogen attacks (Robinson et al., 1997). Therefore, the better understanding of these gene structures may provide the potential to increase the resistance of existing *V. vinifera* commercial cultivars through genetic modification by transformation with introduced genes through gene pyramiding programs.

Ano et al. (2003) reported the characterization of a class III chitinase gene from *V. vinifera* cv. Koshu and demonstrated the antifungal activity of *Chi3K* gene *in vitro*. As part of a study of the molecular structure and catalytic activity of other grapevine class III chitinase genes, we report on on the cloning of *VvChiF* III from *V. vinifera* cv. Flame seedless, the construction of a bacterial expression system for *VvChiF* III and characterization of the hydrolytic activity of the purified VvChiF III protein.

MATERIALS AND METHODS

Plant materials

Leaf samples of *V. vinifera* L. cv. Flame seedless were collected from glasshouse-cultivated cane cuttings, frozen immediately in liquid nitrogen and were stored at -80°C until required.

DNA and total RNA extraction

Genomic DNA was extracted from mature leaves according to Thomas et al. (1993). Isolation of total RNA from uninfected and heavily powdery mildew infected leaf samples was carried out as described by Davies and Robinson (1996). First-strand cDNA was synthesized with a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) from uninfected and infected leaves followed by hydrolysis of total RNA with RNase H.

PCR amplification of *VvChiF* III

Two oligonucleotide primers, *VvChiF* III-F primer, 5'-TCCCTCGAGACATACACGTTATTCAAG-'3 (added *Xho* I site underlined) and *VvChiF* III-R primer, 5'-TGGTCGACCATTCATCGAGGATGAAGGC-3' added *Sal* I site underlined) were designed to amplify the *VvchiF* III chitinase gene from *V. vinifera* cv. Flame seedless based on the upstream sequence of *Chi3K* gene (AB105374) and the sequence of acidic chitinases respectively. PCR amplification was performed in a 20 µL total volume containing 1 µL of genomic DNA (10 ng/µL) or 2.0 µL of synthesized cDNA, 1X High Fidelity PCR buffer, 1.5 mM MgSO4, 200 µM of each dNTPs, 0.5 µM of each primers and 1 U Platinium Taq High Fidelity. PCR amplifications was performed in a thermocycler (iCycler, BioRad, USA) with an initial denaturing step of 94°C for 2 min, followed by 30 amplification cycles of 94°C for 30 sec, 60°C for 30 sec, 68°C for 60 sec and a final extension cycle at 68°C for 5 min. PCR products were electrophoresed on a 1.2% TBE agarose gel, stained with ethidium bromide and visualized on a UV transilluminator (Bio-Doc It, UVP Co., USA).

Cloning and sequencing the *VvChiF* III

PCR products were extracted and purified using QIAquick Gel DNA Extraction Kit protocol (Qiagen, CA). Purified PCR products were ligated in to pGEM-T Vector (Promega) according to the manufacturer's instructions. Plasmid extraction performed using QIAprep Spin Miniprep Kit (Qiagen, CA). Sequencing was performed on a AB 3730xl DNA sequencer. DNA sequencing reactions were carried out using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Norwalk, CT, USA) according to the manufacturer's instructions. Sequences were analyzed using various basic local alignment search tools (BLAST) served at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of pGEX-4T-3/*VvChiF* III for expression in *E. coli*

After confirmation of the *VvChiF* III sequence, the cloned fragment was excised from pGEM-T/*VvChiF* III by digestion with *Xho* I and *Sal* I and ligated into similarly cut glutathione S-transferase (GST) fusion vector pGEX-4T-3 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) to make the recombinant plasmid (pGEX-4T-3/*VvChiF* III).

GST fusion protein expression and purification

For expression of GST-*VvChiF* III, *E. coli* JM109 cells harbouring pGEX-4T-3/*VvChiF* III were grown overnight in LB-broth with 50 µg/mL ampicilin to OD_{600}= 1.0 before induction with 1 mM IPTG at 20°C overnight. After induction of GST fusion protein expression, *E. coli* cells were collected by centrifugation and washed twice in 10 mM Tris-HCl buffer (pH 7.5), containing 100 mM NaCl, 1 mM EDTA and 1 mM DTT. Cells were resuspended in the same buffer and then lysed by sonication. The GST-VvChiF III fusion protein was separated from the sonicated extract with a Glutathione-Sepharose 4B column (Amersham Pharmacia Biotech AB) as described by Harper and Speicher 1997. GST-tag was cleaved with thrombin protease. The rVvChiF III was purified according to Ano et al. (2003).

SDS-PAGE

Recombinant proteins from the *E. coli* JM109 cells harbouring pGEX-4T-3/*VvChiF* III were analysed by SDS-PAGE according to Laemmli (1970) using 15% polyacrylamide gels followed by staining with coomassie brilliant blue.

Enzyme assays

Chitinase activity was assayed with glycolchitin as a substrate. Ten microliters of the sample solution were added to 500 µl of 0.2% (w/v) glycolchitin in 0.1 M sodium acetate buffer (pH 4.0). After incubation of the reaction mixture at 37°C for 30 min, the reducing power of the mixture was measured with ferri-ferrocyanidine reagent according to Imoto and Yagishita (1971).

Antifungal activity assay

Pure rVvChiF III was tested for fungal growth inhibitory activity on *B. cinerea* according to Ano et al. (2003) and by a microspectrophotometric method (Broekaert et al., 1990). A spore suspension (50 µL of 5×10^4 spores/mL in 1X potato dextrose broth)

and sample solution (50 µL of 0-200 µg/mL in resuspension buffer) were combined into each well of a 96 well microtitre plate. The microplate was incubated for 48 h at 25°C and the absorbance at A_{600} nm measured.

RESULTS AND DISCUSSION

A single PCR product of approximately 1Kb was amplified from genomic DNA of *V. vinifera* cv. Flame seedless using primers *VvChiF* III-F and *VvChiF* III-R. PCR products of the same size were also amplified from cDNAs prepared from total RNA extracted from uninfected and powdery mildew-infected leaves (Figure 1). The level of transcript appeared to be markedly higher in the infected leaf tissue. Sequencing of the cloned PCR products confirmed that the PCR products were identical indicating the absence of an intron. A Web-BLAT search of grapevine genome sequence at Genoscope (www.cns.fr) with the PCR product sequence confirmed it encoded a *VvChiF* III with a single open reading frame located on chromosome 16. The sequence has been submitted to GenBank with the accession number EU935006.

The nucleotide sequence of *VvChiF* III and the deduced amino acid sequence are shown in Figure 2. The complete open reading frame is 894 nt in length and encodes a protein of 297 amino acids with a charge of 5.87 at pH 7.0 and isoelectric point of 8.98. This length is comparable to class III chitinases previously isolated from Arabidopsis (Samac et al., 1990), chick pea (Vogelsang and Barz, 1993), rice (Nagasaki et al., 1997) and *V. vinifera* cv. Koshu (Ano et al., 2003).

The amino acid sequence encoded by *VvChiF* III showed a high level of identity to other class III chitinases from grape (Figure 3) including the conserved amino acids in the active-site regions of catalytic domains of family 18 chitinases (Robertus and Monzingo, 1999). VvChiF III was most closely related to Chi3K from *V. vininera* cv. Koshu with a sequence identity of 98%, differing in only three residues 107 (A/V), 215 (P/Q) and 240 (R/Q). However, these residues are not involved in the active-site regions of catalytic domains of family 18 chitinases (Robertus and Monzingo, 1999).

The N-terminal region shares similarity to known signal peptide sequences and may be cleaved from the mature peptide (Hiller et al., 2004). The most likely cleavage site is between position 25 and 26 based on hidden Markov models (Figure 2). Chitinases belonging to family 18 glycosyl hydrolases have two highly conserved regions that are involved in substrate binding and/or catalysis (Fukamizo, 2000). The conserved region II of these chitinases contains several acidic amino acids. The consensus for this region is LDGIDFDIE (L/S) G for plants (Watanabe, 1995). This consensus motif could be seen in the position of 144-154 of amino acids except of a grapevine chitinase (Chit3) in which the sequence is LDGIDFVILLG (protein GenBank ID #CAC14016). Site directed mutagenesis experiments (Manya et al., 1998) and crystallographic data (Perrakis et al., 1994) have shown that the glutamate is involved in the catalytic mechanism and probably acts as a proton donor.

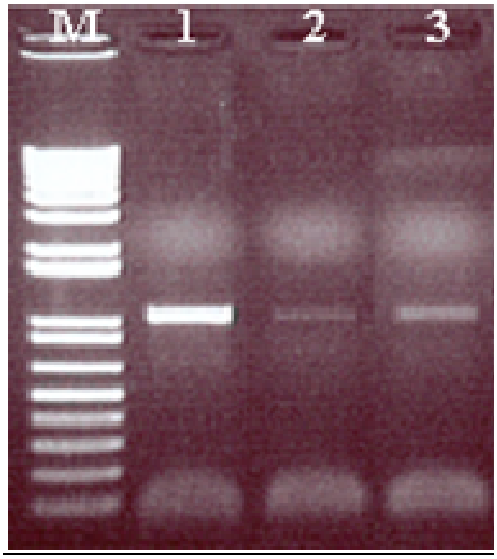

Figure 1. Amplification of *VvChiF* III from *V. vinifera* cv. Flame seedless using gene specific primers with different DNA templates: M, 1 Kb PLUS DNA ladder; Lane 1, genomic DNA; Lane 2, cDNA synthesized from total RNA isolated from uninfected leaves and 3, cDNA synthesized from total RNA isolated from powdery mildew-infected leaves.

These conserved regions may be useful in the design of primers for amplification with the polymerase chain reaction (PCR) or to identify residues that would be appropriate for mutational analyses. Six conserved cysteine residues (Figure 3) in plant class III chitinases are considered to be crucial in maintaining the three-dimensional structure of these proteins (Nagasaki et al., 1997).

Recombinant VvChiF III was expressed in *E. coli* as a GST fusion protein and purified using affinity chromatography and the GST tag cleaved using protease. Purified rVvChiF III was estimated to be a protein of 32 KDa following SDS-PAGE (Figure 4) which is in close agreement with the predicted molecular mass of 31.8 KDa of mature VvChiF III.

Recombinant VvChiF III had the specific activity of 135.9 units per mg toward a soluble substrate, glycol chitin, which was comparative to that of original enzyme (data not shown). Incubation of rVvChiF III with *B. cinerea* spores was observed to have a significant inhibitory effect on fungal growth. The relative growth of *B. cinerea* decreased as the concentration of recombinant chitinase increased in the range 50-200 ug/ml (Figure 5).

The importance of chitinase as a component of a broad-spectrum plant defence mechanism has been well studied (Graham and Sticklen, 1994; Carstens et al., 2003). Sequence analysis indicated that *VvChiF* III

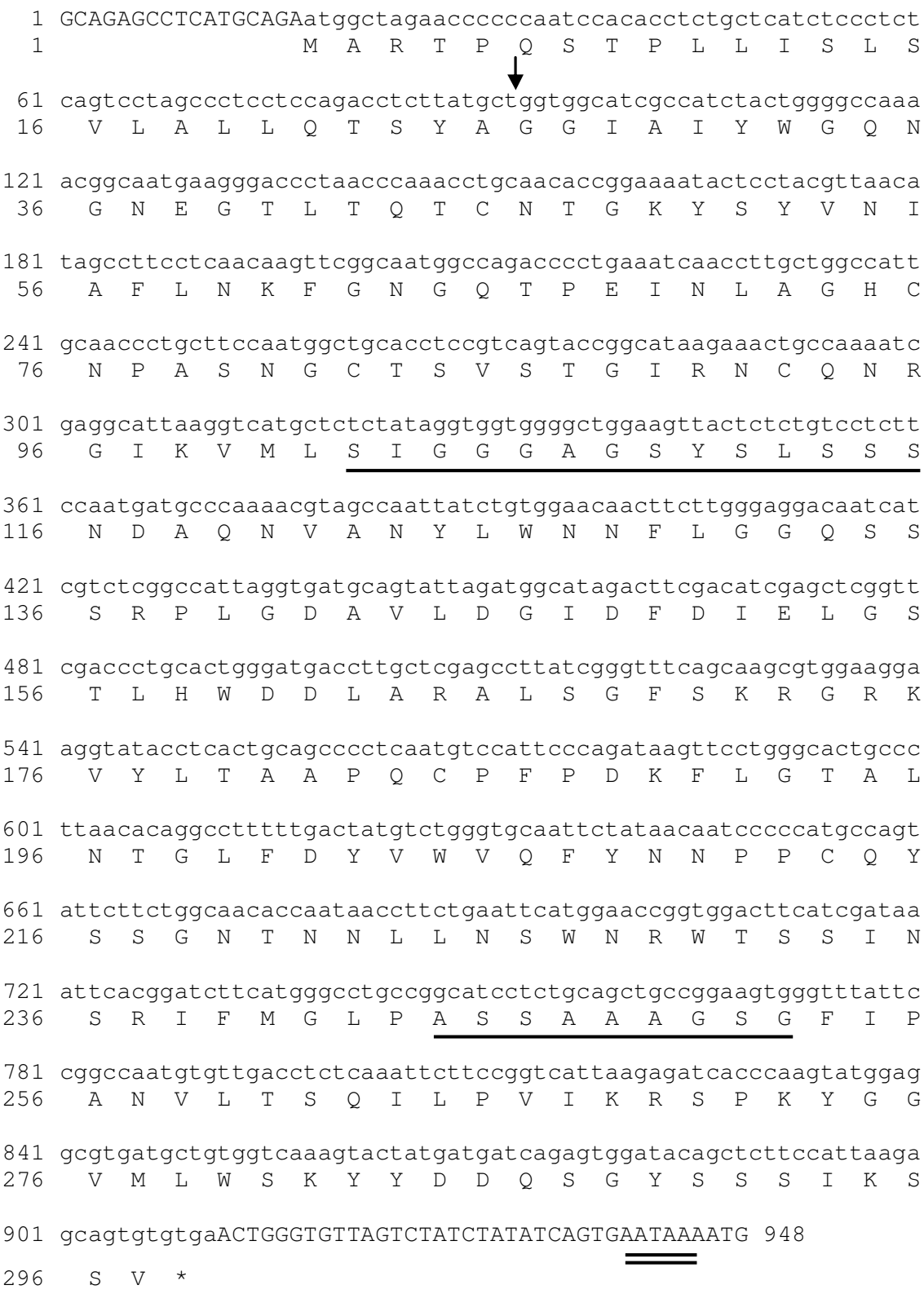

Figure 2. Nucleotide and deduced amino acid sequence of *VvChiF* III gene. Nucleotides are numbered in the 5' to 3' direction, beginning with the first residue of the putative initiation methionine codon ^{18}ATG^{20}. The termination codon TGA is marked with an asterisk. The predicated cleavage position of signal peptide is marked with arrow. Two low compositional complexity regions are indicated by single underlining. A putative polyadenylation signal (AATAAA) is underlined with a double line.

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VvChiFIII : MARTPQSTPLLISLSVLALLQTSYAGGIAIYWGQNGNEGTLTQTCNTGKYSYVNIAFLNKFGNGQ : 65
    Chi3K : ............................................................... : 65
  VVCHIT3 : ............................................................... : 65
    Chit3 : .................F............................................. : 65

VvChiFIII : TPEINLAGHCNPASNGCTSVSTGIRNCQNRGIKVMLSIGGGAGSYSLSSSNDAQNVANYLWNNFL : 130
    Chi3K : ....................................V.......................... : 130
  VVCHIT3 : ............................................................... : 130
    Chit3 : ..........S.........D.S...SQ.........AI....................... : 130

VvChiFIII : GGQSSSRPLGDAVLDGIDFDIELGSTLHWDDLARALSGFSK---RGRKVYLTAAPQCPFPDKFLG : 192
    Chi3K : ...........................................---..................... : 192
  VVCHIT3 : ..................................RIEFQQE..................VP. : 195
    Chit3 : ..R.............V.L....QY...........Q---..................... : 192

VvChiFIII : TALNTGLFDYVWVQFYNNPPCQYSSGNTNNLLNSWNRWTSSINSR-IFMGLPASSAAAGSGFIPA : 256
    Chi3K : .................Q.......................Q-.................. : 256
  VVCHIT3 : ...........................................TGS...........R..... : 260
    Chit3 : ......R..N..A.............T..............-.......A.........P : 256

VvChiFIII : NVLTSQILPVIKRSPKYGGVMLWSKYYDDQSGYSSSIKSSV : 297
    Chi3K : ..................................... : 297
  VVCHIT3 : ..................................... : 301
    Chit3 : ...........T.......................... : 297
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Figure 3. Comparison of predicted amino acid sequence of VvChiF III with three other class III chitinase genes from grapevine: Chi3K (BAC65326), VVCHIT3 (CAA92207), Chit3 (CAC14016). Identical amino acids are indicated by a dot and the dashes indicate gaps. The conserved amino acids in the catalytic domains of family 18 chitinase are indicated in gray. Six conserved cysteine residues are marked with arrows.

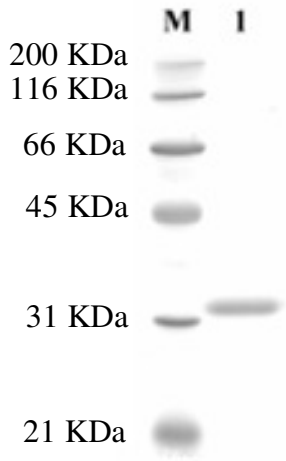

Figure 4. SDS-PAGE of rVvChiF III. The sample was put through SDS-PAGE and stained with Coomassie brilliant blue. M, Marker; lane 1, rVvChiF III.

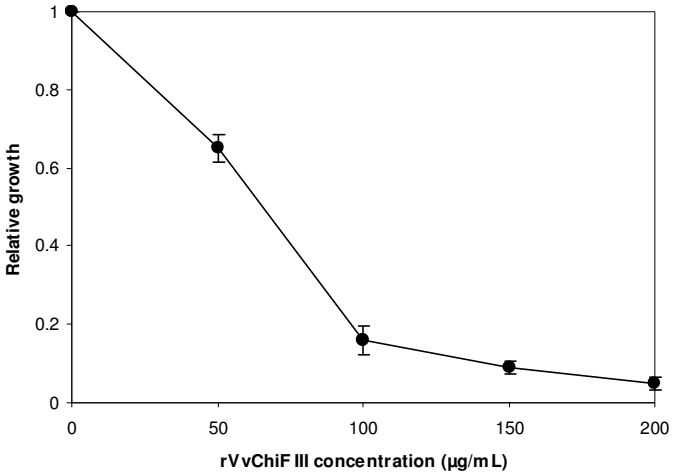

Figure 5. Inhibition of hyphal growth of *B. cinerea* by the rVvChiF III measured by the microspectrophotometric method.

belongs to acidic chitinase class III and the recombinant protein has been demonstrated to have chitinase activity which plays an important role in the defence of grapevines against fungal pathogens by destroy the chitin-

based fungal cell walls, thereby repressing fungal growth. A better understanding of these antifungal grape genes may provide the potential to increase the resistance of existing *V. vinifera* commercial cultivars through genetic modification by transformation with introduced genes through gene pyramiding programs.

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