A Class IV Chitinase Is Highly Expressed in Grape Berries during Ripening

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Chitinase activity increased markedly at the onset of ripening in grape (Vitis vinifera L.) berries and continued to increase throughout the sugar accumulation phase of berry development. In contrast, β-1,3-glucanase activity was not detected in grape berries at any stage of development. Two closely related chitinase cDNAs (VvChiA and VvChiB) were cloned from grapes. Sequence and Southern analysis indicate that these two clones may represent alleles of the same gene. The predicted proteins are acidic and have a signal peptide followed by a cysteine-rich, chitin-binding domain and a catalytic region. An analysis of their sequences indicates that they are class IV chitinases. The deduced protein sequence of VvChiA has a high level of identity with the 32- and 28-kD chitinases present as haze proteins in wine. Expression of VvChiA was high in berries and low in flowers but was not detected in leaves, roots, or seeds. No expression was detected in berries 2 to 8 weeks postflowering, but expression was high 12 to 16 weeks postflowering, which coincided with sugar accumulation and an increase in chitinase activity. Constitutive expression of VvChiA appears to be fruit-specific and induced at high levels in grapes during ripening.

Synthesis of a number of PR proteins is induced in plants following wounding or exposure to pathogens. In general, the genes encoding PR proteins do not show high constitutive levels of expression in plants but are induced in response to pathogens. Fruits, which are rich in sugars and other nutrients, provide an ideal target for pathogens. Resistance mechanisms to fungal pathogens are of particular interest in grapes because of their widespread production for winemaking, dried fruit, and table grapes. Some grape proteins survive vinification and are carried through into wine. These proteins are resistant to proteolysis and precipitate over time, leading to haze in wine. Waters et al. (1996) recently purified the three most abundant proteins in wine and obtained an amino acid sequence that identified them as PR proteins. Two proteins with apparent molecular masses of 32- and 28-kD by SDS-PAGE had homology to chitinases, and a third protein of 24 kD had homology to thaumatin-like proteins. This suggests that these PR proteins are synthesized in grapes and extracted during winemaking and that the solution to wine haze may lie in manipulating viticultural practices that influence the synthesis of these proteins in grape berries. Here we report the cloning of two closely related chitinase cDNAs from grape berries, which are homologous to the 32- and 28-kD wine haze proteins. Expression of these genes is fruit-specific and is induced at high levels throughout the sugar accumulation phase of ripening in grapes.

Two PR proteins that have a significant role in the defense against invading fungal pathogens are chitinase and β-1,3-glucanase. These two proteins hydrolyze chitin and β-1,3-glucans, respectively, which are abundant in the cell walls of many fungi, and the enzymes have been shown to exhibit antifungal activity in vitro (Mauch et al., 1988; Arlorio et al., 1992). Enhanced resistance to fungal pathogens has also been demonstrated in transgenic plants overexpressing chitinase or β-1,3-glucanase, with a synergistic benefit where both genes are present (Jach et al., 1995; Jongedijk et al., 1995). There have been few investigations of chitinase and β-1,3-glucanase in fruit tissues. Esaka et al. (1993) found that levels of chitinase were higher in pumpkin (Cucurbita pepo L.) fruit than in leaves, stems, or seeds, and Derckel et al. (1996) recently reported that chitinase activity in grape (Vitis vinifera L.) berries was also higher than in leaves, roots, or stems.

Abbreviations: *Brix, refractive index measure of total soluble solids; PR, pathogenesis-related.

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**MATERIALS AND METHODS**

**Grape Samples**

Tissue samples from grapes (*Vitis vinifera* L. cv Shiraz) were obtained from John Harvey’s Slate Creek vineyard (Willunga, South Australia) in the 1994/1995 season. Grapes at the mid-ripe stage were also collected from the same vineyard for cv Riesling (14.8 °Brix) and cv Semillon (17.0 °Brix). Grapes were also harvested from the Waite vineyard (Urrbrae, South Australia) for cv Muscat Gordo Bianco (13.5 °Brix) and cv Sultanina (22.7 °Brix) and from the vine-type collection at Nuriootpa, South Australia, for Pinot Noir (21.6 °Brix) and Chardonnay (21.3 °Brix). Cabernet Sauvignon (15.5 °Brix) grapes were collected from Mac Cleggett’s vineyard (Langhorne’s Creek, South Australia). All grape samples were of sound fruit with no detectable symptoms of disease or damage. Berries were deseeded and immediately frozen in liquid nitrogen and stored at −80°C until required. Total soluble solids (°Brix) were measured with a hand-held refractometer (model 10430, Reichert, Vienna, Austria).

**Cloning Grape Chitinase Genes**

Isolation of total RNA from cv Shiraz grapes (harvested 10 weeks postflowering), purification of mRNA, and synthesis of first-strand cDNA using an oligo(dt)-adapter primer were as described by Davies and Robinson (1996). A number of cDNAs encoding chitinase were isolated from the berry cDNA using the rapid amplification of cDNA ends-PCR techniques described by Frohman et al. (1988). Degenerate primers were designed based on the conserved regions of chitinase genes (CHI) and the protein sequences reported for the chitinase proteins that were isolated from wine haze (WHP) by Waters et al. (1996). The forward primers were as follows: CHI7, 5‘-CA(TG)GGIA-TGa/(AG)AA/(TC)TT/(CT)CTAT-3’, corresponding to the amino acid sequence HAGKNF, and WHPI, 5‘-TT(TG)TT(TG)-3’, corresponding to QYYHDYA, and 5‘-GC(TA)TGIA(CA)TGATG-3’, corresponding to FFAH-VTHE. The reverse primers were as follows: CHI3R, 5‘-TGIG(CG)IGCTATCCA/(AG)AACC-3’, corresponding to WFMWTPQ, WHP3R, 5‘-GC/(GA)TA/(GA)T/C/(GA)/TG/(GA)-TA/(GA)TA/(TC)/TG-3’, corresponding to QYYHDYA, and the B25 primer of Frohman et al. (1988) designed to complement the (dT)10-adapter primer.

PCR reactions (25 µL) contained 2.5 µL of cDNA, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% (v/v) Triton X-100, 1.5 mM MgCl2, 200 µM deoxyribonucleotide triphosphates, 1 µM degenerate primers or 0.1 µM B25 primer, and 0.5 unit of Taq DNA polymerase. The PCR conditions were: 2 cycles of 94°C, 1 min; 37°C, 2 min; ramp to 72°C over 2 min; 72°C, 3 min followed by 25 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 3 min; and a final extension of 72°C, 7 min. The reaction products were purified with QiAquick PCR spin columns (Qiagen, Chatsworth, CA) and separated on a low-melting-point agarose gel. Bands were excised and ligated into Bluescript -SK+ plasmid (Stratagene) that had been digested with EcoRV and T-tailed (Marchuk et al., 1990). Twelve independent clones obtained with different primer combinations were sequenced. An analysis of the overlapping sequences allowed the identification of two closely related chitinase cDNAs (VvChi4A and VvChi4B) based on clear differences in the nucleotide sequence. The 5’ ends of these two cDNAs were obtained by PCR from a λ-Zap cDNA library constructed from cv Shiraz grape (10 weeks postflowering) mRNA using a specific reverse primer for the chitinase genes (WHP4R, 5‘-CGTGTGTG-AACATGACGAA-3’) and the T3 or T7 sequencing primers. The identity of the 5’ ends corresponding to the cDNAs was confirmed by comparing the overlapping sequences. All clones were sequenced in both directions by automated sequencing.

**Northern and Southern Analyses**

Blots for northern and Southern analyses were conducted as described by Davies and Robinson (1996). For northern blots, total RNA (4 µg per lane) was separated on a denaturing agarose gel and blotted onto a ZetaProbe membrane (Bio-Rad). Blots were probed with 32P-labeled DNA probes that were prepared by random-primer labeling and washed at high stringency (two washes of 15 min in 0.1X SSC at 65°C) unless stated otherwise.

**Enzyme Extraction**

Frozen leaves and berries were ground to homogeneity in a coffee grinder. Powdered tissue (1 g) was further ground in a mortar and extracted in 10 parts (w/v) of 0.35 m Tris-acetate buffer, pH 8.0, containing 20 mM EDTA, 11 mM sodium diethyldithiocarbamate, 15 mM Cys-HCl, and 6% PEG 4000. The crude homogenate was filtered through two layers of Miracloth (Calbiochem), 0.3 g Polyclar AT (polyvinylpolypyrrolidone, Sigma) g−1 starting leaf material was added, and the mixture was stirred for 15 min. The mixture was cleared by centrifugation at 30,000g for 15 min and the supernatant was desalted on Sephadex G-25 columns (Pharmacia) equilibrated with 10 mM Tris-acetate, pH 7.0, to obtain crude extracts.

**Enzyme Assays**

The activity of β-1,3-glucanase was determined according to the method of Denault et al. (1978) with some modifications. The reaction mixture in a final volume of 1 mL contained 1.5 mg of laminarin, 50 mM sodium acetate, pH 5.2, and an aliquot of extract. The mixture was incubated at 37°C for 20 min and the reducing ends of the hydrolyzed sugars were determined according to the method of Dygert et al. (1965).

Chitinase activity was measured radiometrically with regenerated [3H]chitin. The assay mixture contained 50 mM sodium phosphate, pH 6.5, 1.5 mg of regenerated [3H]chitin prepared as described by Molano et al. (1977), and an aliquot of enzyme in a final volume of 250 µL. The assay was run for 20 min at 37°C in a shaking incubator that was set at 260 rpm. The reaction was terminated with 250 µL of 10% TCA, and after centrifugation at 15,000g for 4 min, radioactivity in 150 µL of the supernatant was determined by liquid-scintillation counting.
RESULTS

Cloning of Grapevine Chitinase cDNAs

Degenerate oligonucleotide primers were designed to correspond to the conserved regions of plant chitinase proteins and to the chitinase peptide sequences for wine haze proteins reported by Waters et al. (1996). These primers were used to amplify cDNA prepared from cv Shiraz grape berries (10 weeks postflowering). Strong bands of the predicted sizes were obtained with five different primer combinations (CHI7 and B25, 820 bp; CH17 and CHI3R, 370 bp; WHPI and B25, 730 bp; WHPI and CHI3R, 280 bp; and WHPI and WHIP3R, 400 bp). Each of these bands was cloned and sequenced. An analysis of the overlapping sequences from the 12 separate clones that were obtained with the different primer combinations indicated the presence of two closely related chitinase genes expressed in ripening grape berries, which we have named VvChi4A and VvChi4B based on the recommendations for naming PR proteins proposed by Van Loon et al. (1994). Clones of VvChi4A were obtained more frequently than were clones of VvChi4B, but at least two independent clones of each were obtained. The 5' ends of the corresponding cDNAs were obtained from a cv Shiraz cDNA library (prepared from mRNA isolated from berries 10 weeks postflowering) by PCR using a gene-specific reverse primer.

The nucleotide sequences of the two cDNAs were 98% identical overall and showed high identity even in the untranslatable regions (data not shown). Many of the nucleotide differences in the coding region did not alter the peptide sequence encoded by the two genes, but VvChi4B had a nine-base insertion near the 5' end, which resulted in an additional three amino acid residues in this protein (Fig. 1). The mature proteins predicted from the two VvChi4 cDNAs were 96% identical. The derived protein sequences of VvChi4 cDNAs had a high degree of similarity to the peptide sequences of both the 32- and 28-kD chitinases reported by Waters et al. (1996), although there were some minor differences (Fig. 1). The differences may relate to varietal differences, since the cDNA clones were obtained from cv Shiraz grapes and the proteins were purified from wine made from cv Muscat Gordo grapes.

An analysis of the VvChi4 sequences using the PSORT program (Nakai and Kanehisa, 1992) indicated that the proteins had a 20-residue signal peptide at the N terminus and are likely to be targeted to the extracellular space. This program predicts a cleavage site after Ala-20 to give a mature protein with a predicted molecular mass of 25,330 D and a pI of 5.14 for VvChi4A. The 32-kD wine haze protein was blocked at the N terminus, but the N-terminal sequence of the 28-kD wine haze protein reported by Waters et al. (1996) coincides with the deduced sequence of VvChi4A starting at Asp-54 and lacks the three-residue insertion found in VvChi4B (Fig. 1). Cleavage of the VvChi4A protein at this site would yield a mature protein with a predicted molecular mass of 22,100 D. The molecular masses of the 32- and 28-kD wine haze proteins were recently determined by electrospray MS as 25,450 and 22,181 D, respectively (E.J. Waters, personal communication). This is consistent with cleavage at the sites indicated in Figure 1, given the minor sequence differences between the proteins isolated from cv Muscat Gordo wine by Waters et al. (1996) and those isolated from the cv Shiraz clone VvChi4A. These molecular masses are lower than those previously reported for the wine haze proteins (Waters et al., 1996). The 28-kD protein sequence is an N-terminal sequence, whereas the 32-kD sequences are from internal peptide fragments (Waters et al., 1996). The sequences reported by Waters et al. (1996) contained errors, which have been corrected in this figure based on more recent sequence information supplied by E.J. Waters (personal communication).

Chitinases have been divided into four broad classes based on sequence similarity and properties (Graham and Sticklen, 1994). The derived protein sequence for VvChi4A showed a high degree of homology to chitinase sequences, the highest similarity being to the class IV chitinases from maize (accession no. M84164), bean (accession no. X57187), sugar beet (accession no. L25826), and rape (accession no. X61488). An analysis of these protein sequences (Fig. 2) indicates the presence of a signal peptide in which there is virtually no homology between the sequences, followed by a chitin-binding domain containing eight conserved Cys
residues. The chitin-binding domain is separated from the catalytic domain by a hinge region, which is highly variable, and the insertion in VvChi4B is in this region (Fig. 2). The catalytic domain is of a similar size in each of these chitinases. Class IV chitinases are generally smaller proteins and are distinguished by having Cys residues at only seven of the eight positions that are characteristic of the chitin-binding domain of the class I chitinases (Graham and Sticklen, 1994). Based on these characteristics and the homology to other class IV chitinases (Fig. 2) we suggest that VvChi4 is a class IV chitinase.

During the course of our experiments two other cDNA sequences encoding chitinases cloned from a grapevine cell-suspension culture appeared in the GenBank database, a class I chitinase (VvChil, accession no. Z54234) and a class III chitinase (VvChi3, accession no. Z68123), and we have cloned homologous cDNAs from grapevine leaves. The properties of the proteins encoded by these cDNAs are compared with VvChi4A in Table 1. Since all three chitinases have a putative signal peptide, only the sequences and properties of the mature proteins were compared. VvChil and VvChi3 are basic proteins, whereas VvChi4 is acidic. Both VvChil and VvChi4 possess the Cys-rich, chitin-binding domain, but this is absent in VvChi3. VvChil is larger than VvChi3, which in turn is larger than VvChi4. For the predicted mature proteins, VvChi4 shows 66% similarity to VvChil and only 37% similarity to VvChi3. This compares to the similarities of 76 to 79% between VvChi4 and the other class IV chitinases shown in Figure 2, demonstrating a degree of structural conservation between class IV chitinases from different plants.

**Southern Analysis of VvChi4**

A Southern blot of genomic DNA from cv Shiraz grapevines probed with VvChi4A is shown in Figure 3. At low stringency (2X SSC at 65°C) two bands were detected in DNA digested with EcoRI and EcoRV, but in each case only one band was detected at high stringency (0.1X SSC at 65°C). The single band at high stringency (Fig. 3) was consistent with a single locus for VvChi4 in grapevine, and the high degree of similarity between the two sequences suggests that VvChi4A and VvChi4B may be alleles of this locus.

The secondary bands observed at low stringency indicate that another related sequence is also present in the grape genome. A partial sequence of a genomic clone isolated using VvChi4A as a probe showed it to have 62% identity to VvChi4A over 400 bp of the sequence (data not shown). It is not yet clear whether this gene is expressed in grape.

### Table 1. Comparison of predicted mature proteins deduced from three grapevine chitinase cDNAs

<table>
<thead>
<tr>
<th>Property</th>
<th>VvChil</th>
<th>VvChi3</th>
<th>VvChi4A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accession no.</td>
<td>Z54234</td>
<td>Z68123</td>
<td>U97521</td>
</tr>
<tr>
<td>Class</td>
<td>I</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td>Predicted mature protein</td>
<td>E-21 → L-299</td>
<td>G-26 → V-286</td>
<td>Q-21 → C-261</td>
</tr>
<tr>
<td>Residues</td>
<td>279</td>
<td>261</td>
<td>241</td>
</tr>
<tr>
<td>Molecular mass (D)</td>
<td>29,740</td>
<td>27,887</td>
<td>25,331</td>
</tr>
<tr>
<td>pl</td>
<td>7.89</td>
<td>8.33</td>
<td>5.14</td>
</tr>
<tr>
<td>Chitin-binding domain</td>
<td>E-21 → C-56</td>
<td>Absent</td>
<td>Q-21 → C-53</td>
</tr>
<tr>
<td>P-rich hinge region</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Localization</td>
<td>Extracellular</td>
<td>?</td>
<td>Extracellular</td>
</tr>
<tr>
<td>% Amino acid similarity to</td>
<td></td>
<td>37</td>
<td>66</td>
</tr>
<tr>
<td>VvChil</td>
<td>~</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>VvChi3</td>
<td>~</td>
<td>~</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Multiple sequence comparison of chitinase proteins. Residues that are identical in at least three of the sequences are highlighted. The grape sequence is deduced from the VvChi4A cDNA and is compared with chitinases from maize seed (accession no. M84164), bean roots (accession no. X57187), sugar beet leaf (accession no. L25826), and rape cotyledons (accession no. X61488).
Chitinase Expression in Ripening Crape Berries

Figure 3. Southern analysis of VvChi4 chitinase genes in grapevine. Genomic DNA was probed with VvChi4A and washed at low (LOW) stringency (2× SSC at 65°C) or high stringency (HIGH) (0.1× SSC at 65°C).

Expression of VvChi4 in Grapevine

RNA was isolated from different cv Shiraz grapevine tissues and probed with VvChi4A. Although the northern blots were washed under high-stringency conditions (0.1× SSC at 65°C), the high degree of sequence identity between VvChi4A and VvChi4B means that both would be detected by this probe. The VvChi4 genes were highly expressed in the flesh and skin of grape berries and, to a lesser extent, in flowers (Fig. 4), but no expression was detected in the leaves, roots, or seeds even at longer exposures of the blot.

Grapes are a nonclimacteric fruit and show a double-sigmoidal pattern of growth (Coombe, 1992). The berries ripen during the second phase of growth when sugar accumulation, anthocyanin synthesis, and berry softening commence. The onset of this ripening is quite distinct and is termed veraison by viticulturists. cv Shiraz grapes were sampled from a commercial vineyard and showed the normal pattern of development, with veraison occurring 8 weeks postflowering, as indicated by the increase in total soluble solids (°Brix) (Fig. 5A). Activity of chitinase and β-1,3-glucanase were measured throughout berry development (Fig. 5B). Chitinase was not detected in berries before veraison, but there was a significant increase in chitinase activity after veraison that continued throughout ripening. In contrast, β-1,3-glucanase activity was not detected in berries at any stage of development. Using the same extraction and assay procedures we readily detected β-1,3-glucanase activity in grapevine leaves, particularly following wounding (data not shown). This is consistent with the recent report by Renault et al. (1996) of the immunological detection of β-1,3-glucanase in grapevine leaves following infection or wounding.

Northern blots of total RNA extracted from grape berries at different stages of development were probed with each of the three grapevine chitinase genes. Expression of VvChi4 was first detected at 10 weeks and it was highly expressed in berries 12 to 16 weeks postflowering (Fig. 5C). In contrast, VvChi1 and VvChi3 were not detected on blots exposed for the same period (1 h) as VvChi4. Faint bands were detected only after much longer exposures (54 h for VvChi1 and 96 h for VvChi3) for VvChi1 in the 16-week postflowering sample and for VvChi3 in all of the samples.

Figure 5. Chitinase and β-1,3-glucanase activity and expression of chitinase genes during ripening of cv Shiraz grape berries. A, Total soluble solids (°Brix) of grape berries, with the dashed, vertical line indicating the onset of ripening (veraison). B, Chitinase and β-1,3-glucanase activity in deseeded berries. C, Expression of VvChi4 chitinase genes. The blot for VvChi4 was exposed for 1 h, and under these conditions no expression of VvChi1 or VvChi3 was detected.
was extracted from grape berry skins at the mid-ripe stage and probed consistent with the homology to the wine haze proteins. chitinase genes was induced in VvChil expression of the berry cDNA yielded the two closely related chitinase clones, VvChilA and VvChilB. The higher expression observed in some of the other varieties, relative to cv Shiraz, may relate to varietal differences or to the influence of different viticultural practices or environmental factors on the expression of VvChil. The results shown in Figure 6 indicate that the expression is most unlikely to be pathogen-induced and suggests that the onset of expression of VvChil is ripening-related.

**DISCUSSION**

Grapes are a nonclimacteric fruit in which the accumulation of sugar and softening of the berries commences at the onset of ripening, termed veraison (Coombe, 1992). Chitinase activity was not detected in grapes prior to veraison but increased at the onset of ripening and continued to accumulate in the berries throughout ripening. In contrast, β-1,3-glucanase was not detected in grape berries at any stage of development (Fig. 5). Amplification of grape berry cDNA yielded the two closely related chitinase clones, VvChilA and VvChilB. Southern analysis (Fig. 3) indicates that VvChil is most likely encoded by a single gene with two different alleles, VvChilA and VvChilB. The expression of the VvChil chitinase genes was induced in grape berries at veraison and they were highly expressed throughout ripening (Fig. 5C), whereas the expression of two other grapevine chitinase genes, VvChil1 and VvChil3, was insignificant in comparison. This suggests that VvChil is the major form of chitinase in ripe grape berries and is consistent with the homology to the wine haze proteins.

The cv Shiraz grapes used in this study were obtained from a commercial vineyard with excellent disease control procedures, and regular monitoring of the vines gave no indication of disease being present. To confirm that the induction of VvChil observed in cv Shiraz grapes (Fig. 5B) was ripening-related and not coincident with an undetected pathogen infection or climatic event at veraison in this vineyard, RNA was also isolated from grapes of a number of other varieties obtained from three other vineyards, none of which had any detectable disease symptoms. The grapes were all at the mid-ripening stage and all showed high levels of expression of VvChil (Fig. 6). The higher expression observed in some of the other varieties, relative to cv Shiraz, may relate to varietal differences or to the influence of different viticultural practices or environmental factors on the expression of VvChil. The results shown in Figure 6 indicate that the expression is most unlikely to be pathogen-induced and suggests that the onset of expression of VvChil is ripening-related.

The deduced protein sequences of the VvChil cDNAs are very similar to both the 32- and 28-kD wine haze proteins (Fig. 1), which indicates that the 28-kD protein may be derived from the 32-kD form by proteolytic cleavage. The predicted site of cleavage is between the chitin-binding domain and the catalytic region (Fig. 1) and indicates that the chitin-binding domain would be lost in the 28-kD protein. This domain is not necessary for chitinase activity but may alter the properties of the enzyme (Iseli et al., 1993; Graham and Sticklen, 1994). Lange et al. (1996) also found evidence for differential proteolytic processing of a class IV chitinase in bean roots, and cleavage also occurred between the chitin-binding domain and the catalytic region. In grapes it is not yet clear whether this processing occurs in vivo or following extraction of the berries and vinification of the juice.

The VvChil genes were highly expressed in ripening fruit, but expression was not detected in any other grapevine tissues except flowers (Fig. 4). Constitutive expression of chitinase genes is generally found only in roots and floral tissues, and expression in other parts of the plant is induced by pathogens or wounding (Graham and Sticklen, 1994). However, no visible signs of wounding or pathogen attack were detected in this fruit, and expression of VvChil was also detected in a range of healthy fruit of different varieties and from different vineyards (Fig. 6). It seems unlikely that the expression of chitinase was wound- or pathogen-induced in all of these samples. Rather, we believe that the expression pattern observed indicates that these chitinase genes are constitutively expressed in grapes during ripening. A parallel study by Tattersall et al. (1997) showed that the third wine haze protein, a thaumatin-like PR5 protein, was also highly expressed in grapes during ripening. PR proteins do not generally show high constitutive levels of expression, but our observations and those of Fils-Lycaon et al. (1996) and Tattersall et al. (1997) indicate that induction of PR genes during fruit ripening may be a more widespread phenomenon.

Ripening of fruit involves the accumulation of sugars and other nutrients as well as softening and eventual breakdown of cellular structure, all of which would predispose the tissue toward a pathogen attack. A number of defense mechanisms are developmentally induced in germinating seeds (Terras et al., 1992; Vogeli-Lange et al., 1994; Leubner-Metzger et al., 1995) and in floral tissues (Lotan et al., 1989; Wemmer et al., 1994; Harikrishna et al., 1996), which also provide excellent targets for pathogens, and it may be that a similar situation occurs in ripening fruit. Because the tissue is likely to become highly susceptible to pathogens, induction of defense mechanisms could be developmentally rather than
in response to a pathogen. Alternatively, the constitutive expression of PR proteins in these tissues may not be related to pathogen resistance, but these proteins may have a role in normal growth and development.

It is not yet known whether VvChi4 has a role in the defense against fungal pathogens of grapevines. There is evidence that grapes are susceptible to powdery mildew prior to veraison but are resistant thereafter. Delp (1954) reported field and laboratory observations that immature grapes were susceptible to infection, but no new infections occurred on grapes when the soluble solids exceeded 8 °Brix. Chellemi and Marois (1992) exposed healthy grapes at various stages of development to powdery mildew and found that the grapes became resistant to infection above 7 °Brix. Since this coincides with the induction of VvChi4 and the increase in chitinase activity (Fig. 4), synthesis of PR proteins following veraison may contribute to the increased resistance to powdery mildew infection.

Chitinase activity can be induced by a number of chemical signals, including ethylene, ABA, jasmonic acid, and salicylic acid, and chitinase levels can also be influenced by auxins and cytokinins (Graham and Sticklen, 1994). Ripening is induced in climacteric fruit by ethylene, but little is known about the signals that induce ripening in nonclimacteric fruit such as grapes. Endogenous levels of auxin are high in grapes during the early stage of berry development but decline to low levels just prior to veraison and remain low throughout ripening (Coome and Hale, 1973; Cawthorn and Morris, 1982). In contrast, ABA levels in grapes increase markedly at veraison and remain elevated during ripening (Coome and Hale, 1973; Cawthorn and Morris, 1982). The application of endogenous auxins to grapes prior to veraison delays both the increase in ABA and the onset of ripening (Coome and Hale, 1973). It is tempting to speculate that changes in endogenous growth substances in the berry, which induce ripening in grapes, also result in the induction of PR proteins to provide increased resistance to pathogens.

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The GenBank accession numbers for VvChi4A and VvChi4B are U97521 and U97522, respectively.

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