Research Note

Comparison of Grape Chitinase Activities in Chardonnay and Cabernet Sauvignon with *Vitis rotundifolia* cv. Fry

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Abstract: Fungal resistance in *Euvitis* is generally correlated with levels of pathogenesis-related proteins such as chitinase. *Vitis rotundifolia* is resistant to many pathogens that affect *Vitis vinifera*; therefore, grape chitinase activities were compared in *V. rotundifolia* cv. Fry and *V. vinifera* cvs. Cabernet Sauvignon and Chardonnay. Cabernet Sauvignon and Chardonnay chitinase activities were approximately 130-fold and 80-fold higher than Fry activities, respectively. Thus, the high pathogen resistance of Fry may be a result of factors other than chitinase. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Fry berries had two chitinase isoforms, and at least one was not found in the *V. vinifera* berries. Cabernet Sauvignon and Chardonnay had four and five chitinase isoforms, respectively.

Key words: chitinase, muscadine, pathogenesis, protein

Vitis rotundifolia grapevines, commonly called muscadines, are native to the southeastern United States and are resistant to many diseases that affect *Vitis vinifera,* such as *Xylella fastidiosa*, *Botrytis cinerea*, and *Plasmopara viticola* (Galet and Morton 1988, Staudt and Kassemeyer 1995). The most striking physical differences between *V. rotundifolia* and *V. vinifera* are that muscadine grapes are large with thick skins and that they grow in loose clusters of few berries as compared to the thin-skinned, small berries and tight bunches of *V. vinifera*. In addition to physical dissimilarities between the two species that may account for differences in pathogen resistance, *V. rotundifolia* may possess phytoalexins and pathogenesis-related proteins that are absent or less predominant in *V. vinifera*.

The predominant proteins in ripe *V. vinifera* berries, regardless of pathogen presence, are pathogenesis-related proteins consistently identified as chitinases and thaumatin-like proteins (Derckel et al. 1998, Hayasaka et al. 2001, Pocock et al. 2000, Salzman et al. 1998, Tattersall et al. 1997). In comparisons among *Euvitis* cultivars of varying fungal susceptibility, positive correlations between fungal resistance in the field and chitinase activities against synthetic substrates have been observed (Busam et al. 1997, Giannakis et al. 1998, Salzman et al. 1998). Purified grape chitinases have shown antifungal activity in vitro against the grape pathogens *Guignardia bidwellii*, *Botrytis cinerea*, and *Uncinula necator* (Derckel et al. 1998, Giannakis et al. 1998, Salzman et al. 1998), and transgenic grapevines overexpressing chitinase have shown higher resistance to *Botrytis cinerea* than untransformed vines (Kikkert et al. 2000).

Pathogenesis-related proteins in *V. rotundifolia* have not been studied prior to this report, but the correlation between chitinase activity and fungal resistance among *Euvitis* was the basis for the hypothesis that *V. rotundifolia* grapes have higher chitinase activities than *V. vinifera* grapes.

Materials and Methods

Sample collection. Berry samples were collected 2 September 2002 at Martin Vineyards in Knott's Island, NC. Both *Vitis vinifera* cultivars were grown on Couderc 3309 rootstock; *Vitis rotundifolia* cv. Fry was own-rooted. Only ripe, disease-free grapes were used. Separate samples were taken from the southern sides of five randomly selected vines for each variety. For Cabernet Sauvignon and Chardonnay vines, all bunches within 0.9 m to the left of the

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Acknowledgments: The authors thank David Martin for the use of his vineyard and Mark Gay of the University of North Carolina at Wilmington for technical assistance. Bill Cline of North Carolina State University and David Padgett, Thomas Shafer, and Julian Keith of the University of North Carolina at Wilmington are gratefully acknowledged for their encouragement and helpful criticism.

Manuscript submitted March 2004; revised July 2004

trunk were collected, mixed, and three bunches per vine selected blindly as samples. All Fry berries within 0.9 m to the left of each trunk were harvested. *V. rotundifolia* berries ripen at different times; therefore, Fry berries were graded visually and grouped on an arbitrary four point scale: 1, unripe, green berries; 2, unripe, slightly bronze berries; 3, ripe, bronze berries; 4, damaged berries. All berries in group 3 were taken as a single sample per vine. All 15 samples were sealed in plastic bags, frozen immediately on dry ice, and stored at -80°C.

Protein extraction. Deseeded grape tissues were ground in liquid nitrogen with a mortar and pestle to a fine powder that was combined with ice-cold extraction buffer (3 g:10 mL) and mixed gently with a glass stirring rod. Extraction buffer consisted of 100 mM sodium acetate, 0.25% Triton X-100 (Sigma, St. Louis, MO), 20 mM sodium diethyldithiocarbamate (Acros Organics, Pittsburgh, PA), 14 mM ß-mercaptoethanol, and 3.0% polyvinylpyrrolidone (PVPP), cross-linked (Acros Organics), pH 5.0. Shortly after mixing, the grape powder slurries were centrifuged at 30,000 *g* and 4°C for 15 min; supernatants were used as crude extracts. For chitinase activity assays, *V. vinifera* extracts were diluted 100-fold with extraction buffer, excluding PVPP, and Fry extracts were diluted 2-fold so that chitinase activities for both species remained in the linear range of the assay.

Chitinase activity assays. A solubilized, dye-labeled form of chitin, carboxymethyl-chitin-Remazol Brilliant Violet (CM-chitin-RBV), was used as a substrate for colorimetric chitinase activity assays. CM-chitin-RBV was synthesized according to Wirth and Wolf (1990), with modifications. Reagent amounts were scaled proportionally with respect to the starting material, 2.50 g practical grade chitin (Sigma), with the exceptions of chloroacetic acid (5.87 g in 31 mL isopropanol), Remazol Brilliant Violet 5R (Sigma) (1.95 g), Na_2SO_4 (39 g), and $Na_3PO_4 \times 12\ H_2O$ (3.06 g in 12.5 mL deionized water). The resulting CM-chitin-RBV solution was filtered through Whatman 42 filter paper (Whatman International, Maidstone, UK) and the final concentration of CM-chitin-RBV was determined gravimetrically to be 1.5 mg/mL by drying a 5.0 mL sample at 105°C for 16 hr followed by lyophilization for 24 hr.

Chitinase activity assays based on Derckel et al. (1996) were optimized to be linear with respect to time and extract amount and were performed in triplicate. Aliquots of 200 mM sodium acetate buffer, pH 5.0, and CM-chitin-RBV were combined in a ratio of 9:10 (v/v), respectively, and 950 µL of the solution was preincubated at 37°C for 20 min for each assay. Assays were started with the addition of 50 µL dilute extract and stopped after 10 min by the addition of 250 µL 1 N HCl that precipitated undigested substrate. The mixtures were then incubated in an ice bath for 20 hr to facilitate further precipitation and centrifuged at 16,000 *g* for 5 min (Andersen et al. 1997). The supernatants were measured spectrophotometrically at 550 nm and compared against a standard curve of undigested, unacidified CM-chitin-RBV. Chitinase activity was expressed as units per µg protein; one unit equals 1 µg CM-chitin-RBV hydrolyzed per minute (µg CM-chitin-RBV/min).

Protein assays. Extract protein concentrations were determined by an Amido Black (ICN Biomedicals, Aurora, OH) assay according to Weiss and Bisson (2001) using bovine serum albumin (Sigma) to produce a standard curve.

Sugar content and pH determination. Deseeded grapes were thawed, centrifuged at 4°C and 10,000 *g* for 10 min, and the supernatants used for sugar content and pH determination at 20°C. pH was measured with an Accumet 950 pH/ion meter (Fisher Scientific, Pittsburgh, PA); sugar content was measured with a Fisherbrand handheld refractometer (± 0.2 Brix) (Fisher Scientific).

Statistical analyses. Brix, pH, protein concentrations, and chitinase activity as units per µg protein (µg CM-chitin-RBV/[min·µg protein]) were compared using Sigma Stat (ver. 2.0; Jandel Scientific, San Rafael, CA). Differences among varieties were measured with one-way ANOVA. In the case that raw data were not normally distributed, the data were natural logarithm transformed. Varietal differences were considered significant by pairwise multiple comparison procedures (Tukey's test or Duncan's multiple range test) when p was less than 0.05.

Glycol chitin SDS-PAGE. Chitinases isoforms were visualized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 5% stacking gels and 12% acrylamide resolving gels (Laemmli 1970) containing 0.01% glycol chitin synthesized from glycol chitosan (Sigma) according to Trudel and Asselin (1989). *Vitis vinifera* extracts were pooled by variety and dialyzed against 78 mM Tris-HCl, 12.5% glycerol (v/v), 0.0013% bromphenol blue (w/v), pH 6.8 in 6000 to 8000 MWCO tubing (Spectrum Laboratories, Rancho Dominguez, CA). Fry extracts were pooled and concentrated about 12-fold by dialyzing against dry polyethylene glycol compound (Sigma, MW 15,000 to 20,000) before dialysis against the same buffer. Dialyzed samples were concentrated with 10,000 MWCO Microsep centrifugal devices (Pall Life Sciences, Ann Arbor, MI), adjusted to 2% (w/v) SDS from a 10% stock solution, and boiled for 5 min. Gels were run at room temperature in a Hoefer Mighty Small II SE-260 system (Pharmacia Biotech, San Francisco, CA). After electrophoresis, gels were incubated for 13 to 20 hr at 37°C in 200 mL 100 mM sodium acetate, 1% Triton X-100 (v/v), pH 5.0 to renature proteins and promote chitinase activity against glycol chitin. The gels were stained with Fluorescent Brightener 28 (Sigma) and bands of chitinase activity visualized over a UV transilluminator according to Trudel and Asselin (1989). Gels were photographed with a Camedia C-3000 digital camera (Olympus America, Melville, NY) and photograph contrast was enhanced using Paint Shop Pro (ver. 7.02; JASC Software, Eden Prairie, MN). Molecular weights of proteins corresponding to bands of glycol chitin hydrolysis were determined by comparison to low range, prestained SDS-PAGE standards (BioRad, Hercules, CA).

Results

Chitinase activity, sugar content, pH, and extract protein concentration data are shown in Table 1. *Vitis rotundifolia* cv. Fry extracts had significantly higher protein concentrations (Tukey's test: $p < 0.05$) than Chardonnay but not Cabernet Sauvignon. Duncan's multiple range test ($p < 0.05$) indicated that Chardonnay and Cabernet Sauvignon extract protein concentrations were not significantly different and that Fry extracts had significantly more protein than both *V. vinifera* cultivars.

Raw data for chitinase activity per µg protein were not normally distributed for at least one cultivar; therefore, activity data were natural logarithm transformed to produce normal distribution for each variety. There were significant differences among all three varieties in chitinase activity per µg protein calculated using Tukey's test ($p < 0.05$). Fry had the lowest activity, Chardonnay activity was approximately 80-fold higher than Fry, and Cabernet Sauvignon activity was approximately 130-fold higher than Fry.

The average sugar content of the Fry samples was significantly lower than the values for Cabernet Sauvignon and Chardonnay. The Brix of the samples in this study differ from typical values for ripe grapes most likely because heavy rain fell shortly before sample collection. However, pH data are in the range of expected values for ripe grapes. Ripe *V. rotundifolia* grapes have sugar contents that range from 10 to 18 Brix; pH ranges from 3.0 to 3.5. Therefore, the significant differences among the low sugar content of the Fry berries versus the *V. vinifera* berries coincide with published observations (Carroll et al. 1991).

Chitinase isoforms from grape extracts separated by SDS-PAGE are shown in Figure 1. Molecular weights estimated from prestained standards are presented in Table 2. Cabernet Sauvignon and Chardonnay extracts displayed chitinase isoforms of similar molecular weights at 39 and 26 kDa. The 36, 34, and 31 kDa isoforms in Chardonnay consistently appeared to be unique. The 38 kDa isoform in Fry appeared to be unique throughout four SDS-PAGE trials under similar conditions. The 30 kDa Fry isoform appeared to be the same molecular weight as the corresponding Cabernet Sauvignon isoform, but not the 31 kDa Chardonnay isoform.

Discussion

The crude berry extracts of *V. rotundifolia* cv. Fry had significantly lower chitinase activity than the berry extracts of Cabernet Sauvignon and Chardonnay. The lower activities of Fry can be explained in two ways: Fry berries may contain lower constitutive concentrations of chitinase than the *V. vinifera* berries or the specific activity of chitinase in Fry is lower; that is, chitinase isoforms in Fry are less active against CM-chitin-RBV than *V. vinifera*

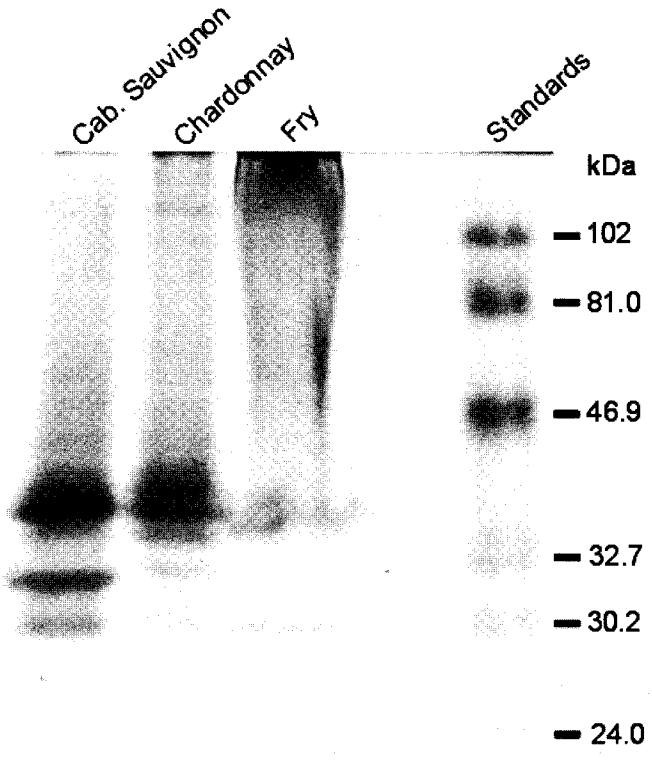

Figure 1 Separation of chitinase isoforms by glycol chitin SDS-PAGE. Lanes 1, 2, and 3 were loaded with approximately 3, 4, and 30 µg protein, respectively.

Table 1 Average data for extract protein concentration, chitinase activity, sugar content (Brix), and pH for the three varieties (± 1 standard deviation, n = 5). One unit of chitinase activity is equivalent to 1 mg CM-chitin-RBV hydrolyzed per minute (mg CM-chitin-RBV/min).

Cultivar	Protein concn (µg/mL)	Chitinase activity units/mg protein	Brix	pH
Cabernet Sauvignon	65.2 ± 17.2	203 ± 16.8	17.6 ± 1.3	3.75 ± 0.07
Chardonnay	64.8 ± 18.7	123 ± 40.8	17.5 ± 1.1	3.98 ± 0.07
Fry	90.4 ± 6.5	1.54 ± 0.53	12.0 ± 1.0	3.23 ± 0.06

Table 2 Estimated molecular weights (MW) of chitinase isoforms associated with chitinase activity after glycol chitin SDS-PAGE.

MW (kDa)	Cabernet Sauvignon	Chardonnay	Fry
39	x	x	
38			x
36		x	
34		x	
33	x		
31		x	
30	x		x
26	x	x	

isoforms. Although it is possible that artificial chitinase assays are not ecologically representative indicators of antifungal capabilities, correlations between chitinase activity against artificial substrates and fungal resistance in *Euvitis* have been demonstrated (Giannakis et al. 1998, Salzman et al. 1998). The correlation between chitinase activity and pathogen resistance holds true in the comparison of Cabernet Sauvignon and Chardonnay berries in this study. Cabernet Sauvignon, which had significantly higher chitinase activity than Chardonnay, is considered a fairly fungi-resistant *V. vinifera* cultivar and Chardonnay is not, particularly with respect to late season rots (Carroll et al. 1991). Based on the extremely low chitinase activity in Fry berries, it is unlikely that constitutively produced chitinase is accountable exclusively for the high fungal resistance of Fry; therefore, other mechanisms of resistance should be considered.

Dai et al. (1994) and Giannakis et al. (1998) found that the intensities and speeds of pathogenesis-induced responses correlated with fungal resistance among *Euvitis* varieties. It is possible that Fry has lower levels of chitinase constitutively, but that, upon pathogen recognition, induced chitinase concentrations increase faster and equal or exceed the induced levels of *V. vinifera* chitinase. Additionally, some plant chitinases with low activity are believed to act as signaling molecules for induced responses; upon fungal infection, they release chitin oligomers that elicit additional antifungal activities (Kasprzewska 2003). The role of constitutive chitinases in the defense of *V. rotundifolia* may be part of a signal transduction pathway and there may be inducible chitinase isoforms. The localization of chitinase in *V. rotundifolia* berries remains undetermined and may differ from the distribution of chitinase activity in *V. vinifera* berries, where Derckel et al. (1998) found 98% of chitinase activity in the pulp. It is possible that *V. rotundifolia* berries contain chitinase activity in the skin that induces antifungal defenses and inhibits spore germination and fungal growth before it reaches the pulp.

Chitinases are thought to be somewhat pathogen specific; some isoforms may be effective against particular fungi species and ineffective against others (Busam et al. 1997, Giannakis et al. 1998). Therefore, *V. rotundifolia* chitinase isoforms may be more effective against some pathogens than *V. vinifera* isoforms. By SDS-PAGE, Fry had at least one unique 38 kDa chitinase isoform and one that appeared to have the same molecular weight as a 30 kDa Cabernet Sauvignon isoform. No chitinase isoforms from Chardonnay had the same apparent molecular weights as Fry and only two of the five Chardonnay isoforms had molecular weights that coincided with Cabernet Sauvignon.

The estimated molecular weights in this study ranged from 26 to 39 kDa as compared to grape chitinase molecular weights of 31 to 34 kDa determined by SDS-PAGE (Derckel et al. 1998, Pocock et al. 2000). Generally, molecular weights of grape chitinases determined by SDS-PAGE are higher than values determined by mass spectrometry that range from 25.3 to 25.9 kDa (Hayasaka et al. 2001, Pocock et al. 2000). In addition to the anomalous migrations typical of grape chitinases during SDS-PAGE, the molecular weights in this study could have been influenced by the inclusion of glycol chitin in resolving gels. Although the protein samples were denatured by boiling with SDS, some affinity toward glycol chitin may have remained, resulting in reduced migrations and artificially high observed molecular weights.

In studies of *V. rotundifolia* proteins by SDS-PAGE, Lamikanra (1987) found predominant 32 and 24 kDa proteins; Mazhar et al. (2002) suggested that a major 30 kDa muscadine protein could be chitinase. Our results suggest that constitutive *V. rotundifolia* berry chitinases are either minor proteins and, therefore, do not correspond with predominant SDS-PAGE bands in *V. rotundifolia* general protein studies, or that constitutive *V. rotundifolia* chitinases, if indeed major proteins, have very low specific activities compared to those of *V. vinifera*.

Although only three varieties were used in this study, the approximately 100-fold difference in activity between *V. rotundifolia* cv. Fry and the *V. vinifera* varieties suggests that similar differences in chitinase activity between the two species are likely even if not at such a magnitude. The disparity in chitinase activity between the two species suggests that they have evolved different strategies for fungal defense not only physically but also biochemically. Probably, differences in pathogen resistance between the two species are influenced by factors in addition to chitinase activity such as other pathogenesis-related proteins, phytoalexins, cluster architecture, and skin properties.

Conclusion

The abundance of chitinases in *V. vinifera* berries at ripeness, regardless of injury, suggests that they are involved in fungal defense. In this study, only ripe, disease-free grapes were used. Therefore, the results of this study are more relevant to constitutive defenses against late-season diseases, such as *Botrytis cinerea*, rather than diseases such as *Uncinula necator* that colonize grapes preveraison. Chitinase activity at different developmental stages and induced chitinase expression should be studied to further elucidate the fungal resistance strategies of *V. rotundifolia*. Knowledge of the biochemical differences between the host-pathogen interactions of *V. rotundifolia* and *V. vinifera* will be useful in understanding pathogen defense strategies among grapes, in improving cultivation practices, and in designing grape breeding programs and transgenic grapevines.

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