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Identification of a *Vitis vinifera* endo- β -1,3-glucanase with antimicrobial activity against *Plasmopara viticola*

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SUMMARY

Inducible plant defences against pathogens are stimulated by infections and comprise several classes of pathogenesis-related (PR) proteins. Endo- β -1,3-glucanases (EGases) belong to the PR-2 class and their expression is induced by many pathogenic fungi and oomycetes, suggesting that EGases play a role in the hydrolysis of pathogen cell walls. However, reports of a direct effect of EGases on cell walls of plant pathogens are scarce. Here, we characterized three EGases from *Vitis vinifera* whose expression is induced during infection by *Plasmopara viticola*, the causal agent of downy mildew. Recombinant proteins were expressed in *Escherichia coli*. The enzymatic characteristics of these three enzymes were measured *in vitro* and *in planta*. A functional assay performed *in vitro* on germinated *P. viticola* spores revealed a strong anti-*P. viticola* activity for EGase3, which strikingly was that with the lowest *in vitro* catalytic efficiency. To our knowledge, this work shows, for the first time, the direct effect against downy mildew of EGases of the PR-2 family from *Vitis*.

Keywords: endo- β -1,3-glucanase, grapevine, *Plasmopara viticola*, PR-2, *Vitis vinifera*.

INTRODUCTION

Plants possess defences against pathogens involving an array of molecular mechanisms (Danilova, 2006; Hatsugai and Hara-Nishimura, 2010; Jones and Dangl, 2006). The first line of defence is a physical and chemical barrier, comprising the cuticle (Hückelhoven, 2007; Reina-Pinto and Yephremov, 2009), lignin deposits on cell walls, phytoalexins (Ahuja *et al.*, 2012; Jeandet *et al.*, 2002) and antimicrobial proteins (Cândido *et al.*, 2011). However, these compounds do not protect against all pathogens. A second line of defence comprises receptor-mediated defences that are induced following pathogen recognition, such as cell wall modifications (Hückelhoven, 2007), the release of reactive oxygen species (ROS) (Desikan *et al.*, 1998; Mittler *et al.*, 2011), the accumulation of secondary metabolites and the synthesis of proteins

known as pathogenesis-related (PR) proteins (van Loon *et al.*, 2006; Stintzi *et al.*, 1993). The last line of defence is the hypersensitive response, leading to cell death (Hara-Nishimura and Hatsugai, 2011; Hofius *et al.*, 2011).

PR proteins are synthesized by plants in response to pathogen attack and their synthesis is induced by signalling compounds, such as jasmonate, salicylate or ethylene (van Loon *et al.*, 2006). They comprise an array of proteins with different functions, such as oxidases, chitinases and protease inhibitors. Based on their function, PR proteins are classified into 17 families, from PR-1 to PR-17 (van Loon *et al.*, 2006; van Loon and van Strien, 1999). The PR-2 class is composed of endo- β -1,3-glucanases (EGases) (Balasubramanian *et al.*, 2012), which are widespread enzymes in plants. They are not only involved in defence responses, but also in many physiological functions, such as pollen development (Worrall *et al.*, 1992), germination (Morohashi and Matsushima, 2000), cell division and elongation (Ebrahim *et al.*, 2011), and fruit ripening (Leubner-Metzger, 2005).

Plant EGases are grouped into several classes, according to their amino acid sequence, structural properties and subcellular localization (Davies and Henrissat, 1995). Class I EGases have been described as basic proteins, localized in the vacuole (Bulcke *et al.*, 1989). They generally harbour an N-terminal signal peptide and a short glycosylated C-terminal sequence for vacuolar targeting. Class II, III and IV EGases are usually acidic, deprived of the C-terminal sequence (Bulcke *et al.*, 1989; Kauffmann *et al.*, 1987) and are generally secreted by default in the apoplast. Several EGase transcripts are induced on infection (Jin *et al.*, 2007; Neuhaus *et al.*, 1992; Ward *et al.*, 1991), but their expression level can also change during plant development (Balasubramanian *et al.*, 2012). EGases hydrolyse β -1,3-glucosidic bonds in β -1,3-glucans, which are structural components of the cell walls of pathogenic fungi and oomycetes (Adams, 2004; Wessels, 1994). EGases are thus believed to directly hydrolyse pathogen cell walls, causing the lysis of pathogen cells (Britto *et al.*, 2013). Accordingly, enzymes belonging to Class I have been reported to inhibit the growth of fungi *in vitro* (Arlorio *et al.*, 1992; Ebrahim *et al.*, 2011). EGases also contribute to plant defence by producing oligosaccharide elicitors that trigger the synthesis of other PR-compounds (Klarzynski *et al.*, 2000), such as phytoalexins (Keen *et al.*, 1983).

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Although numerous investigations have described the role of EGases in plant defence, only a few have described a direct effect of EGases on a pathogen. Here, we describe the cloning, protein purification and enzymatic analysis of three EGases from *Vitis vinifera* whose expression is induced on infection by *Plasmopara viticola*, the causal agent of grapevine downy mildew. We show that the enzyme displaying the lowest catalytic activity *in vitro* has the strongest effect on *P. viticola* germinated spores. Our results provide evidence of the effect of *V. vinifera* EGase activity on pathogen viability and suggest that care must be taken when interpreting the results of *in vitro* experiments with artificial substrates.

RESULTS

Selection of *V. vinifera* EGases and enzyme classification

The protein sequences of two full-length, mRNA-derived EGases induced by pathogen infection (accession numbers *AAF4467* and *CAB91554*) were used to search the grapevine genome for similar sequences. We retrieved a total of 31 proteins with strong similarity to either of the two query proteins (BLASTP, E value $\leq 10e-40$). The alignment of these proteins, the query sequences and sequences of EGases from Arabidopsis, tobacco and soybean allowed the identification of eight potentially misannotated or truncated proteins that were excluded from downstream analysis, which resulted in the phylogenetic tree shown in Fig. 1a. It is worth noting that all the EGases that are involved in responses to biotic stress in species other than grapevine cluster together in a clade of the phylogenetic tree.

Glucanases involved in defence are expected to be either vacuolar or apoplastic. We thus searched for proteins having a predicted signal peptide and whose expression was confirmed by the existence of the corresponding cDNA, yielding seven proteins (Fig. 1a, green and asterisk). Investigation of the RNA-Seq data revealed that two of the seven genes (*VIT_208s0007g06040*, *VIT_18s0122g00980*) were expressed in berries, two genes (*VIT_08s0007g05990*, *VIT_08s0007g06000*) were non-responsive to treatments and three genes (see below) were induced to different degrees in response to *P. viticola* infection (Table S1, see Supporting Information).

The three predicted proteins whose expression was induced on *P. viticola* infection were selected for detailed biochemical analysis and are referred to hereafter as EGase1 (*VIT_205s0077g01150*), EGase2 (*VIT_206s0061g00120*) and EGase3 (*VIT_208s0007g06060*). All three proteins belong to the clade containing the biotic stress-induced proteins in Fig. 1a. An alignment of the three proteins is presented in Fig. 1b. Pairwise identity between EGases varies from 57% to 60%, with amino

acid similarity ranging from 72% to 76%. Enzyme characteristics deduced from databanks are shown in Table 1.

The three EGases from *Vitis* belong to the GH17 family and to the GH-A clan (Lombard *et al.*, 2014). They have the same EC number (EC 3.2.1.39) and thus are likely to display similar enzymatic properties. They have close theoretical molecular weights, but whereas EGase1 and EGase3 have theoretical basic pI values of 9.5 and 9.39, respectively, EGase2 has an acidic theoretical pI value of 4.93. According to the pI values and the data from the literature (Bulcke *et al.*, 1989; Ebrahim *et al.*, 2011), EGase1 and EGase3 are considered to be Class I endoglucanases and are predicted to be localized in the vacuole. However, it should be noted that only EGase1, but not EGase3, exhibits a C-terminal vacuolar targeting sequence (Fig. 1b). EGase2 is predicted to be a Class II glucanase with an apoplastic localization.

Analysis of gene expression

To verify that all three EGases are expressed, we studied their expression in grapevine leaves during *P. viticola* infection by means of semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). As presented in Fig. 2a, the expression of EGase2 and EGase3 was induced at 24 h post-infection (hpi) by *P. viticola*, simultaneous with the detectable increase in pathogen growth, as shown by the increase in pathogen actin expression. Meanwhile, EGase1 expression remained low and was weakly induced by infection. All three EGases were weakly expressed in grapevine leaves mock inoculated with water, with a transient induction observed for EGase2 and EGase3 at 72 hpi (Fig. S1, see Supporting Information).

In parallel, we compared the expression of all three EGases *in silico* in two independent RNA-Seq datasets involving *P. viticola* infection. EGase3 expression was strongly induced by infection in both datasets and showed the highest basal expression level, EGase1 was weakly induced by *P. viticola* infection in both datasets and, finally, the EGase2 induction level was dependent on the dataset (Fig. 2b,c; Table S1).

Enzyme purification, activity and kinetic parameters

The results of protein purification are shown in Fig. 3. All EGases were obtained with purity higher than 95%. The molecular weights measured on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for EGase1, EGase2 and EGase3 were 39, 34 and 36 kDa, respectively, close to the predicted molecular weights of the mature proteins (38.5, 35 and 35 kDa, respectively), showing that no observable proteolysis occurred during purification.

The specific activities of the EGases were measured at 37°C, using laminarin as a substrate (Table 2). EGase2 had the highest specific activity (35.2 ± 1.3 nkat/nmol); the specific activities of EGase1 and EGase3 (17.2 ± 1.6 and 3.4 ± 0.2 nkat/nmol,

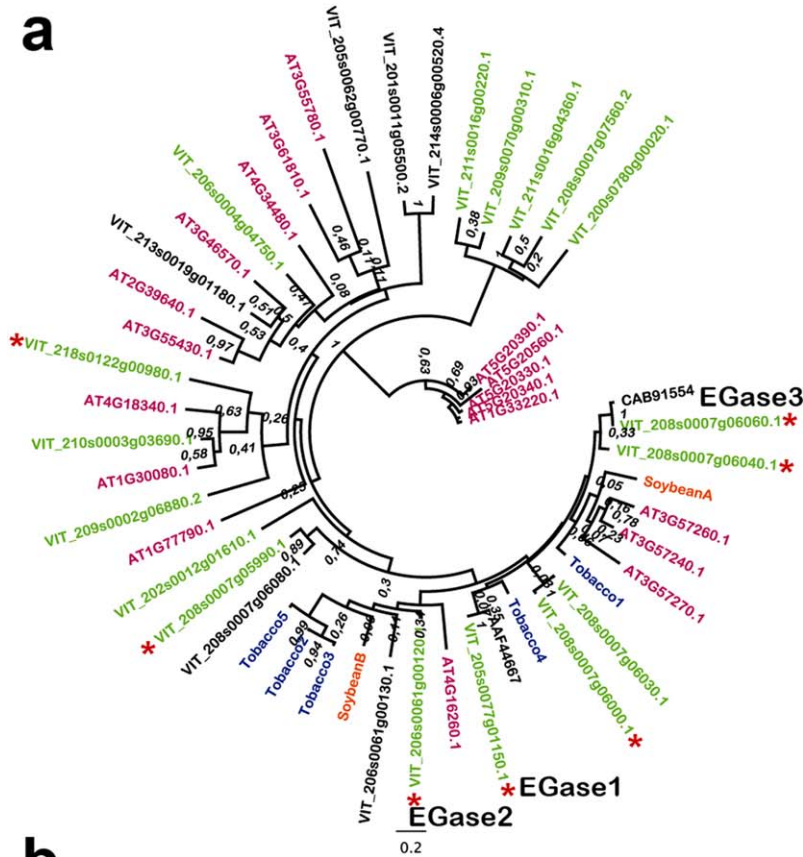


Fig. 1 Analysis of grapevine endo-β-1,3-glucanase (EGase) sequences. (a) Phylogenetic tree containing grapevine (black and green), Arabidopsis (magenta), tobacco (blue) and soybean (orange) endoglucanases. Grapevine sequences in green indicate proteins with a predicted signal peptide. Asterisks indicate grapevine genes for which evidence of expression is available. Numbers on the nodes indicate bootstrap values. Sequences selected as EGase1, EGase2 and EGase3 are specified. Grapevine and Arabidopsis endoglucanases are named according to the Grapevine Genome Database (v2) at CRIBI and The Arabidopsis Information Resource (TAIR), respectively (see Experimental procedures). The accession numbers in the tree correspond to the two original sequences used in the BLAST search. Accession numbers for tobacco and soybean glucanases are as follows: Tobacco1, CAA38324; Tobacco2, AAA34079; Tobacco3, AAD33880; Tobacco4, AAA34078; Tobacco5, AAA34103.1; SoybeanA, AAA33946; SoybeanB, AAR26001. (b) Alignment of the three selected grapevine endoglucanases and a tobacco endoglucanase possessing a vacuolar targeting signal. The black background shows identical amino acid residues. The grey background represents similar amino acids. Asterisks indicate predicted catalytic glutamic acid (Glu) residues. Blue bars show the divergent region in the vicinity of a catalytic Glu. Red bars show the glycosyl hydrolase family 17 signature.

b

Tobacco4	1	--MDHSHKHIALQMAAILLLGLTVSSTETVGAQSVGVCYGM LGNNLPPASQVVO LYKSKHN
EGase1	1	-----MAAVLLLGFLAALQITGVKSVGVCYGM LGNNLPPASQVVO LYKSKRN
EGase2	1	-----MALTRNRPVVLVLLGFLVIMSTITIGAQSVGVCYGRNNGNLPASQVVO LYKSKNG
EGase3	1	MAMFDSTVTKSSRMATLLVFLGGLMASLHLTVAQTGVGVCYGM EDNLPFGQVVOGLVNGYS
Tobacco4	59	IRRMRLYDPNQAALQALRGSNIEVMLGVPNSDLONIAANPSNANNVVQRNVRNFWPAVKF
EGase1	49	IDRMRIYDPNQAALQALRGSNIEVMLGVPNSDLQGLANPSQAQSNVQRNVRNFWPQVVF
EGase2	56	IGSMRIYDPNSDLQALRGSNIEVMLGVPNSDLQSLASDASAAATVQNVVNVYASEVKF
EGase3	61	IRRMRLYDPNQAALQALRGSNIEVMLGVPNDALQNIAGIQGNANSVQVNNIKNYG-NVRF
Tobacco4	119	RYIAVGNEVSPVTC-TSLSLTRYLLPAMRNIRNAISSAGLQNNIKVSTSDVDMTLIGNSQPP
EGase1	109	RYIAVGNEVSPVNGGTSRFAQVLPAMRNIRNALASAGLQDRVKVSTAIIDLTLGNSYPP
EGase2	116	RYIAVGNEVLPVTCG-NAQVAQVLPAMRNIRNAISSAGLQDQIKVSTATPFAVLGRSYPP
EGase3	120	RYIAVGNEVSPVTC-----PTAQVLPAMRNIRNAISSAGLQNIQVSTAIQVGLGNSYPP
Tobacco4	178	SGGSRFNDVRSFIDPIITGVIRGINSPLLVNIIPYFSYAGNPRDISLPYALFTAPNVVVQD
EGase1	169	SGGARFQDVRGYLDPIITGFVLDVNGSPLLANIIPYFSYSGNPKDISLPYALFTANSVVVVQD
EGase2	175	SGGSRFDDVSGFIIPIISFIAENKSPLLANIIPYFSYVGDQNRDLQYALFTASGVVVQD
EGase3	176	SRGARFKEVTSFLLNPIISFLVNRAPLLVNIIPYFSYVGNPRDISLPYALFTAPNVVVQD
Tobacco4	238	GS LGYRNLFDAMLDVYVAALSRAGGSLHIVVSESGWPSAGGAFATVNNAAATYKNILOH
EGase1	229	GQRYKYNLFDAMLDVYVAALSRAGGSLHIVVSESGWPSAGGFTVVDNARTYKNILOH
EGase2	235	GSYQYQNLFDAMLDVYVAALSRAGGSLHIVVSESGWPSGGGAFATVVDNARTYKNILOH
EGase3	236	GQ LGYRNLFDAMLDVYVAALSRAGGSLHIVVSESGWPSAGGAFATVVDNARTYKNILOH
Tobacco4	298	VKRGSPRRPNKVIETYLFAFDENKKN- FELEKHFGLFSPNKQPKYPLSGFGFSDRYWDMS
EGase1	289	VKGGTPKRPGRVETYLFAFDENKKN- FOLEKHFGLFSPNKQPKYSINFSG-EKPDWDS
EGase2	295	VKGGTPKRSGRVETYLFAFDENKKN- FELEKHFGLFSPNKQPKYPLSGFGFSDRYWDMS
EGase3	296	VKGGTPKRPKGGPIETYLFAFDENKKN- FEVEKHFGLFSPNKQPKYPLSGFGFSDRYWDMS
Tobacco4	357	AENNATAASTLISEM
EGase1	347	SENDTNEESLNSDI
EGase2		-----
EGase3		-----

Table 1 Biochemical characteristics of the three endo- β -1,3-glucanases (EGases) from *Vitis*.

	EGase1	EGase2	EGase3
Gene	VIT_205s0077g01150	VIT_206s0061g00120	VIT_208s0007g06060
Accession no.	Q9M563	A7PQW3	F6HLL8
GH family number	GH17	GH17	GH17
Clan	GH-A	GH-A	GH-A
Three-dimensional structure status	(β/α)8	(β/α)8	(β/α)8
E.C.	3.2.1.39	3.2.1.39	3.2.1.39
Residues	376	344	345
MW (Da)	41286.04	37092.64	37501.89
pI	9.5	4.93	9.39
Catalytic Glu	132; 278	123; 268	127; 269
Predicted mature protein	39–278	27–268	27–269

Accession number is from UniprotKB. GH family number, clan, three-dimensional structure status and E.C. are from the CAZy (carbohydrate-active enzymes database) site (<http://www.cazy.org>). Molecular weights (MW) and pI were determined from the enzyme sequences. Catalytic glutamic acid (Glu) residues were predicted using PROSITE.

respectively) were approximately one-half and one-tenth of that of EGase1, respectively.

The kinetic parameters of the three enzymes were also measured using laminarin as a substrate and the results are shown in Table 2. The Michaelis constant K_M approximates the affinity of the substrate for the enzyme (and is considered to be an apparent dissociation constant); the turnover constant k_{cat} defines the maximum number of substrate molecules converted to product per unit of time; the specific constant k_{cat}/K_M combines the effectiveness of reaction with the effectiveness of substrate binding.

The K_M values showed that EGase3 had the highest affinity ($81 \pm 2.5 \mu\text{M}$) for the substrate, followed by EGase1 ($147 \pm 15 \mu\text{M}$) and EGase2 ($228 \pm 25 \mu\text{M}$). Based on the turnover constant (k_{cat}) values, EGase2 was the most efficient enzyme to transform substrate into product ($92 \pm 7.0 \text{ s}^{-1}$), followed by EGase1 ($34 \pm 3.0 \text{ s}^{-1}$) and the least efficient enzyme EGase3 ($3 \pm 0.2 \text{ s}^{-1}$).

The specificity constant (k_{cat}/K_M) of EGase2 had the highest value, followed by EGase1 and EGase3. Overall, the specific activities and specificity constants for the three EGases revealed the same relative efficiency for the three enzymes: EGase1 exhibited around one-half of the EGase2 activity and EGase3 showed 10% of the activity of EGase2.

The differences in activity are surprising because all three enzymes show a high level of sequence identity. It should be noted that, apart from the vacuolar targeting signal, the most significant differences between enzyme sequences are located at the N-terminus and in the vicinity of one of the predicted catalytic residues (Fig. 1b).

Substrate specificity and effect of pH and temperature on glucanase activity

In order to establish enzyme specificity, the activity of EGases was assessed on several substrates (see Experimental Procedures). The production of reducing sugars could not be detected using these

substrates, suggesting a narrow substrate specificity of the three enzymes for β -1,3-branched sugars.

To evaluate the effect of pH, the activity of the three EGases was measured at 37°C, at pH values ranging from 3.0 to 7.5, with 0.5 increments. The results are presented in Fig. 4. EGase1 had a maximum activity at pH 5.5, whereas the maximum activity for both EGase2 and EGase3 was achieved at pH 5.0. Taken together, the three enzymes are active in the same pH range, with a wider pH range for EGase2.

The effect of temperature on activity was also assessed. As shown in Fig. 4, the optimum temperature was around 40°C for EGase1 and EGase2, and higher, around 50°C, for EGase3. It is worth noting that the relative activity of EGase3 at 60°C is 80% of its maximum activity, compared with only 20% for EGase1 and EGase2.

Enzymatic activity of *in planta*-expressed glucanases

To evaluate the enzyme activity when expressed *in planta*, the three EGases were transiently expressed in *Nicotiana benthamiana* leaves by agroinfiltration. Glucanase activity was measured in leaf protein extracts at 2 days post-infiltration using laminarin as a substrate. The results of enzyme activity *in planta* recapitulated the results obtained *in vitro* (Fig. 5).

Effect of glucanases on germinated *P. viticola* spores

In order to evaluate the potential role of EGase1, EGase2 and EGase3 in the defence against pathogens, we studied their effect on *in vitro*-germinated spores of *P. viticola*. To do so, germinated spores were incubated with each of the three enzymes, and the viability and integrity of zoospores were evaluated over time.

Observations were performed at 60-s intervals for 12 min following enzyme addition. The results of spore counting under transmitted or fluorescent light at 0 and 12 min are shown in Fig.

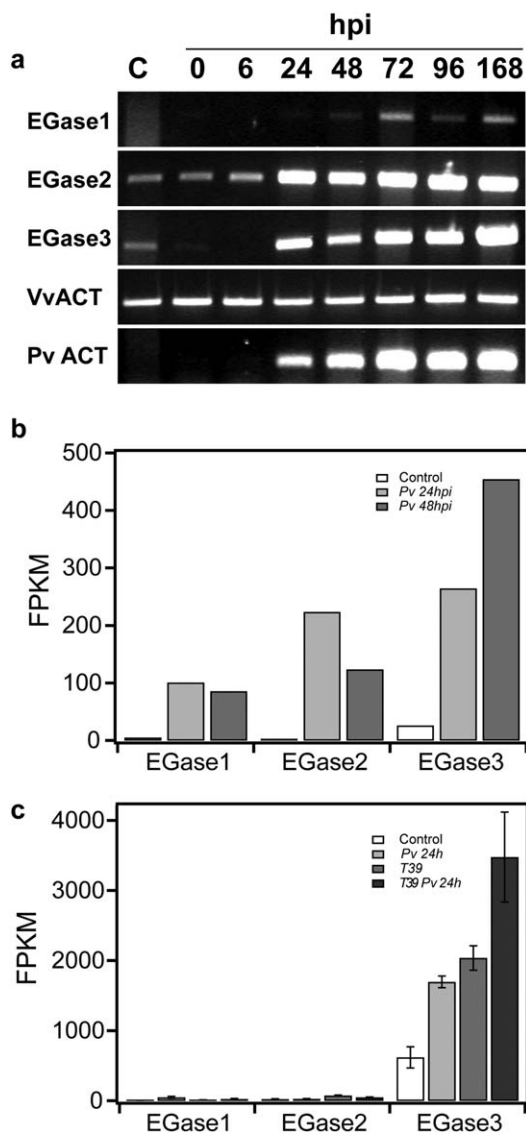


Fig. 2 Expression of grapevine endoglucanases on *Plasmopara viticola* infection. (a) Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) showing the expression of grapevine endo- β -1,3-glucanases (EGases) at 0, 6, 24, 48, 72, 96 and 168 h post-inoculation (hpi). Non-inoculated leaves were used as control (C). PvACT, *Plasmopara viticola* actin; VvACT, *Vitis vinifera* actin. The results are representative of two independent experiments. (b) Expression of EGases on grapevine leaves based on RNA-Seq data from Vannozzi *et al.* (2012). *Pv 24hpi*, *P. viticola*-infected leaves at 24 hpi; *Pv 48hpi*, *P. viticola*-infected leaves at 48 hpi; FPKM, fragments per kilobase of exon per million fragments mapped. (c) Expression of EGases on grapevine leaves based on RNA-Seq data from Perazzolli *et al.* (2012). *Pv 24h*, *P. viticola*-infected leaves at 24 hpi; *T39*, leaves treated with *Trichoderma harzianum* T39, which induces resistance to downy mildew; *T39 Pv 24h*, *P. viticola*-infected leaves following treatment with T39 at 24 hpi; FPKM, fragments per kilobase of exon per million fragments mapped. Data shown are the means and standard errors of three biological replicates.

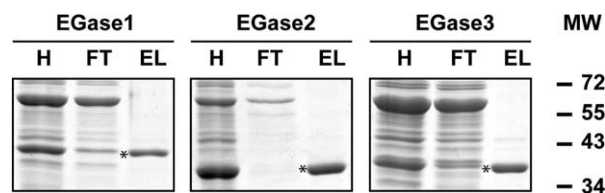


Fig. 3 Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified grapevine endoglucanases. H, soluble lysate containing recombinant endo- β -1,3-glucanase (EGase); FT, flow-through of affinity Ni-TED column; EL, purified recombinant EGase, indicated by an asterisk. Two micrograms of each enzyme were run on a 12% acrylamide gel and stained with colloidal Coomassie blue. MW, molecular weight.

Table 2 Kinetic parameters of the three endo- β -1,3-glucanases (EGases) from *Vitis vinifera*.

	Activity (nkat/nmol)	K_M (μM)	k_{cat} (s^{-1})	K_{cat}/K_M ($\mu\text{M}^{-1} \text{s}^{-1}$)
EGase1	17.2 ± 1.6	147 ± 15.0	34 ± 3.0	0.23
EGase2	35.2 ± 1.3	228 ± 25.0	92 ± 7.0	0.4
EGase3	3.4 ± 0.2	81 ± 2.5	3 ± 0.2	0.04

6 and Table S2 (see Supporting Information). Suspension buffer had little effect on the viability of germinated spores. EGase2 did not show any important effects on the survival and integrity of germinated spores. The presence of EGase1 resulted in a decrease in living spores, without a noticeable change in spore structural integrity (Fig. 6b,c). Finally, the addition of EGase3 resulted in an important decrease in both the number of living spores and structural integrity (Fig. 6b,c).

DISCUSSION

Here, we report the expression analysis, cloning and biochemical characterization of three EGases from *V. vinifera* that we named EGase1, EGase2 and EGase3.

Expression of EGase2 and EGase3 mRNAs was clearly induced on *P. viticola* infection, and the induction paralleled the expression of *P. viticola* actin, suggesting a link between pathogen presence and the induction of EGase2 and EGase3. EGase1 expression was weaker, but its induction was confirmed by inspection of RNA-Seq data. Based on the accession numbers provided by the authors, EGase1 has been reported to be induced by *Botrytis cinerea* (Bézier *et al.*, 2002), laminarin (Aziz *et al.*, 2003) and UV light (Bonomelli *et al.*, 2004). The results of induction of EGase3 on infection by *P. viticola* were also confirmed by the analysis of two independent sets of RNA-Seq data (Fig. 2b,c, Table S1). Based on the accession numbers provided by the authors, EGase3 has been reported to be induced by ethephon (Jacobs *et al.*, 1999), by infection with *Erysiphe necator*, the causal agent of powdery mildew (Jacobs *et al.*, 1999), by the non-host oomycete *Pseudoperonospora cubensis* (Kortekamp, 2006) and by *P. viticola* (Kortekamp,

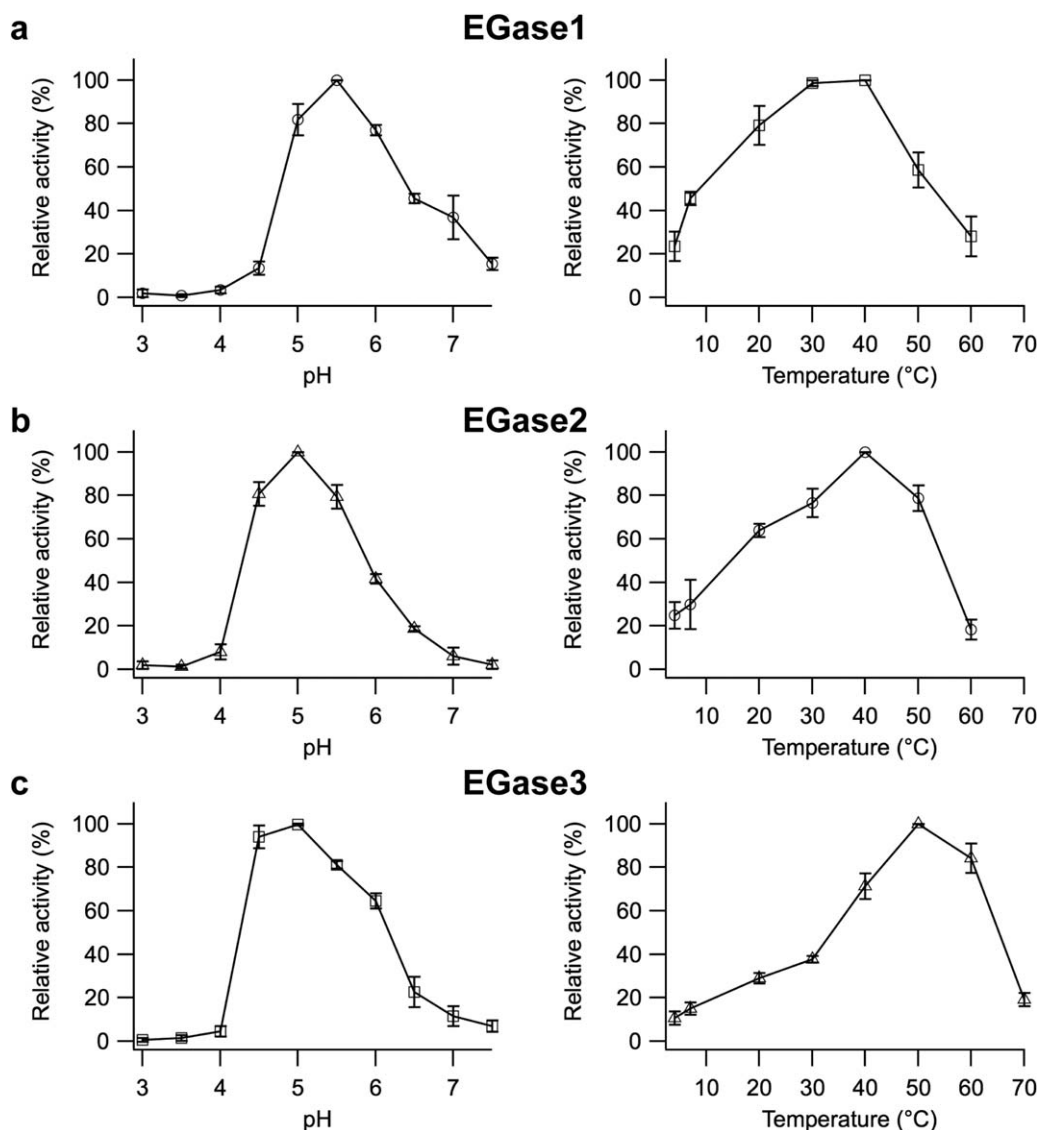


Fig. 4 Effect of pH and temperature on grapevine glucanase enzymatic activity. Effect of pH and temperature on the activity of EGase1 (a), EGase2 (b) and EGase3 (c). Effect of pH (left panels) was measured at a constant temperature of 37°C. Effect of temperature (right panels) was measured at a constant pH of 5.4. The pH of the buffer was not affected by temperature. In both cases, the activity is expressed relative to the maximum activity of 100%. Data are the means of three independent experiments with no technical repetitions, and the error bars represent the standard deviation.

2006), although induction in the latter was observed in a resistant species, but not in a susceptible cultivar. Finally, inspection of available RNA-Seq data revealed different levels of induction for EGase2 on *P. viticola* infection depending on the dataset. Our results are thus in agreement with previous reports.

Basic EGases, such as EGase1 and EGase3, have been commonly classified as Class I and considered to be localized to the vacuole. However, EGase3 lacks a C-terminal vacuolar sorting signal. There are other examples of basic EGases lacking a vacuolar sorting C-terminal extension (Liu *et al.*, 2010; Xie *et al.*, 2015) suggesting that they are not necessarily vacuolar. Indeed, Xie

et al. (2015) reported ZmGns, a maize basic EGase that lacks a vacuolar targeting signal and shows antimicrobial activity. Intriguingly, the closest relatives to ZmGns in grapevine are EGase1 (53% identity, 93% alignment cover) and EGase3 (48% identity, 98% alignment cover).

In vitro enzymatic assays revealed that the enzymatic activity and kinetic constants showed important differences between all three EGases, results that were confirmed when using EGases expressed *in planta* (Fig. 5). Values obtained for K_M were similar to those measured for three basic EGases from barley (Hrmova and Fincher, 1993), but the k_{cat} values calculated in this study

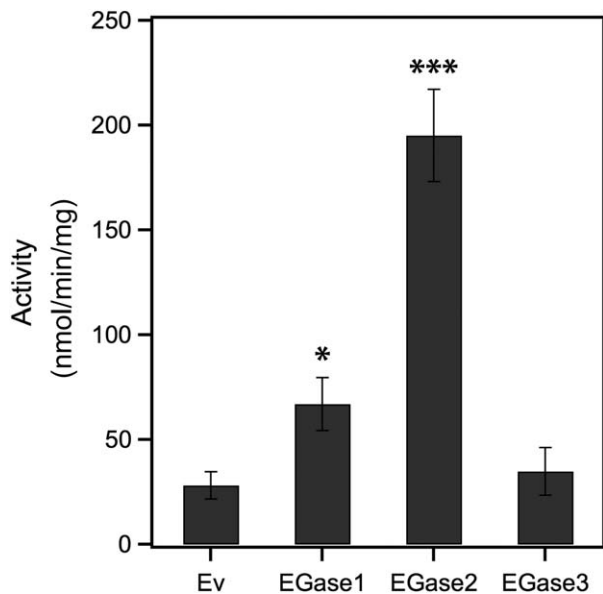


Fig. 5 Activity of grapevine endoglucanases produced *in planta*. Glucanase activity in crude extracts of *Nicotiana benthamiana* leaves transiently expressing grapevine endoglucanases. Plants agroinfiltrated with an empty vector (Ev) were used as controls. Mean value and standard error were obtained from three biological repetitions, each including two technical repetitions. Asterisks indicate samples significantly different from Ev in a Dunnett's test at * $P = 0.1$ and *** $P = 0.01$.

were lower. One reason for the low catalytic efficiency and differences in the catalytic parameters could be differences in substrate specificity. However, enzymatic assays performed with several substrates with various proportions of β -1,3 links did not shed any light on this matter. Concerning pH and temperature dependence, the values found here were in agreement with the optimal temperatures for endoglucanases (Hrmova and Fincher, 1993; Kikuchi *et al.*, 2005). The optimum pH values for EGase1, EGase2 and EGase3 were pH 5.5, pH 4.5 and pH 5, respectively. Interestingly, the pH and temperature dependence of EGase3 were very similar to those for ZmGns (Xie *et al.*, 2015), suggesting possible functional similarity. It is tempting to speculate that the large range in pH and temperature dependence of the three EGases may provide adaptive function(s) of GH-17 EGases to different environmental, biotic or abiotic conditions.

Despite showing the highest activity *in vitro*, EGase2 did not show any antimicrobial activity against *P. viticola* germinated spores. A possible function for EGase2 could be callose degradation. Callose is a high-molecular-weight β -1,3-glucan that is commonly induced following pathogen challenge. Although mainly considered as a way to reinforce the cell wall and thus prevent pathogen penetration, callose also acts as a negative regulator of salicylic acid (SA)-mediated responses (Nishimura *et al.*, 2003; Oide *et al.*, 2013). Accordingly, plant EGases have been reported to possess callose-degrading activity and to influence the outcome of different plant–pathogen interac-

tions (Beffa and Meins, 1996; Oide *et al.*, 2013). In particular, Oide *et al.* (2013) reported a pathogen-induced acidic glucanase with callose-degrading activity, which led us to speculate that EGase2 could be involved in callose degradation and, consequently, activate SA-mediated responses. Alternatively, EGase2 could be involved in the degradation of endogenous molecules whose products could act as elicitors of defence responses (Klarzynski *et al.*, 2000), which have been proven to be efficient against *P. viticola* infection in grapevine (Aziz *et al.*, 2003).

EGase1 and EGase3 showed a behaviour opposite to EGase2. EGase1 and, especially, EGase3 showed antimicrobial activity against *P. viticola* germinated spores, despite presenting the lowest activity *in vitro* against laminarin. We can rule out the possibility that this observation is caused by a contaminating activity in the protein extracts (e.g. chitinase or protease), as all EGases were produced in the same bacterial strain and purified using identical procedures. The differences in activity could be explained if branching surrounding the β -1,3 bonds is more favourable to EGase3 activity in the context of a cell wall than inside laminarin. Alternatively, the antimicrobial activity could be independent of glucanase activity; rather than hydrolysing the cell walls, EGase3 could interfere with cell wall synthesis and lead to pathogen lysis. A better knowledge of the composition of zoospores, and experiments to determine whether these structures are lysed by EGases, would shed light on this matter. Whatever the mechanism behind the antimicrobial activity of EGase3, the fact that it was the grapevine EGase showing the strongest increase in expression following *P. viticola* infection (Fig. 2b,c; Perazzolli *et al.*, 2012; Vannozzi *et al.*, 2012) supports its direct role in defence against *P. viticola*. Furthermore, EGase3 is strongly induced by treatment with the ascomycete *Trichoderma harzianum* strain T39 (T39), a biocontrol agent that induces resistance to *P. viticola* (Perazzolli *et al.*, 2012). T39-induced resistance to *P. viticola* could thus be explained, in part, by the antimicrobial activity associated with a higher level of EGase3 expression.

The most obvious function for plant EGases is the degradation of cell walls from fungal and oomycete pathogens, which are composed of different amounts of β -1,3-glucans. Accordingly, the expression of EGases is induced on pathogen attack (Jin *et al.*, 2007; Ward *et al.*, 1991). Efforts to achieve disease resistance by the overexpression of EGases have produced variable results (Balasubramanian *et al.*, 2012; van Loon *et al.*, 2006) and, in most cases, they showed little or no effect of glucanases on pathogenicity. Altogether, reports of a direct lytic effect of glucanases on plant pathogens are scarce; other than this report, Xie *et al.* (2015) reported an effect of ZmGns on the germination of *Aspergillus flavus*, whereas Arlorio *et al.* (1992) showed changes in fungal cell wall morphology associated with the presence of a combination of chitinases and glucanases, although the main effect was caused by chitinases. At this point it is worth noting the multiple similarities between maize ZmGns and grapevine

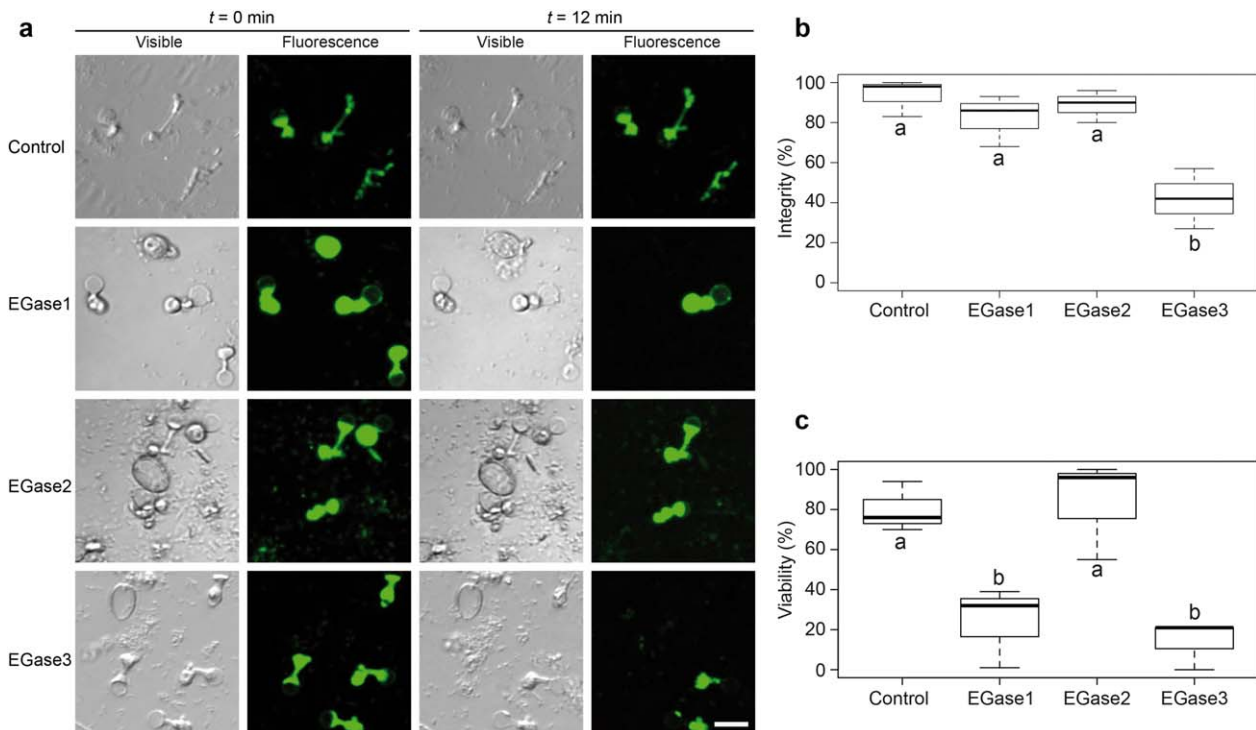


Fig. 6 Effect of endoglucanases on germinated *Plasmopara viticola* spores. (a) Glucanase assay on *P. viticola* germinated spores. Each line represents a different treatment, where purified endo- β -1,3-glucanases (EGases) or buffer as control were added to the spore suspension. The results of each treatment are illustrated by a photograph of the same microscopic field taken at different times ($t = 0$ min and $t = 12$ min) under two different light sources. Fluorescent light reveals fluorescein diacetate (FDA) fluorescence and indicates live zoospores, whereas visible light allows the counting of structurally intact zoospores. The effect on spore viability can be observed for all three EGases (fluorescence panels). The effect of treatment on spore integrity is visible for EGase3. Bar represents 15 μ m. (b) Percentage of spores showing structural integrity after 12 min of treatment. Percentages were calculated by counting spores at $t = 0$ min and $t = 12$ min in visible light. (c) Percentage of spore viability after 12 min of treatment. Percentages were calculated by counting spores at $t = 0$ min and $t = 12$ min in fluorescent light. In (b) and (c), boxplots show the results of three independent experiments without technical repetitions. Boxes with the same letter are not significantly different in a Tukey's honestly significant difference (HSD) test at $P = 0.05$. Raw data for spore counting are detailed in Table S2.

EGase3, which prompted us to propose that the two enzymes are functional homologues.

In summary, we have reported the cloning and characterization of three grapevine EGases of the GH-17 family. Their enzymatic characteristics suggest a wide and complementary range of action. The expression of EGase2 and EGase3 was induced on *P. viticola* infection, but only EGase3 displayed strong antimicrobial activity with only poor *in vitro* activity. To the best of our knowledge, this is the first report of the lytic effect of a plant endoglucanase on a plant pathogenic oomycete.

Oomycetes use glucanase-inhibiting proteins (GIPs) to block the action of plant EGases and to colonize plants (Rose *et al.*, 2002). The identification of the *P. viticola* GIP inhibiting EGase3 will allow the performance of structural research to understand the molecular basis of the interactions between the two proteins. This knowledge will, in turn, open up the way to the use of gene-editing technologies to modify the structure of EGase3 in order to avoid interaction with GIP, whilst maintaining enzymatic activity, which should result in increased resistance to *P. viticola*.

EXPERIMENTAL PROCEDURES

Ampicillin, laminarin, tris(hydroxymethyl)aminomethane (Tris), 3(*N*-morpholino)propanesulfonic acid (MOPS) and fluorescein diacetate (FDA) were obtained from Sigma-Aldrich (St Louis, MO, USA). Tris-glycine-SDS buffer (10 \times), acrylamide (37.5/1) and pre-stained molecular weight markers were obtained from Euromedex (Souffelweyersheim, France). The Protino Ni²⁺ tris-carboxymethyl ethylene diamine (Ni-TED) 150 kit was obtained from Macherey-Nagel (Düren, Germany). Ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail was purchased from Roche (Basel, Switzerland). LR and BP clones were obtained from Life Technologies-Thermo Fisher Scientific (Waltham, MA, USA). *Escherichia coli* Arctic Express strain was obtained from Agilent Technologies (Santa Clara, CA, USA). The MiniPrep kit was purchased from QIAGEN (Hilden, Germany).

Plant material

Seedlings of *V. vinifera* cv. Muscat Ottonel, which is susceptible to downy mildew, were grown on stone wool in a glasshouse at 22°C/19°C (day/night) with a photoperiod of 16 h of light. *Nicotiana benthamiana* plants

were grown in a glasshouse at 22°C/18°C (day/night) with a photoperiod of 14 h of light.

Phylogenetic and sequence analyses

Grapevine EGase sequences were identified by BLAST against the proteome deduced from the *V. vinifera* line 40024 genome sequence (Jaillon *et al.*, 2007) using two known grapevine EGases (accession numbers AAF4667 and CAB91554). Proteins were retrieved from the Grape Genome database (v2) at CRIBI (Vitulo *et al.*, 2014). *Arabidopsis thaliana* EGases were obtained by performing a search on The Arabidopsis Information Resource (TAIR, <http://www.arabidopsis.org/>) using the term 'family 17', which produced 57 entries. Visual inspection of the output revealed 47 EGases. The 18 EGases predicted to be apoplastic were included in the analysis. Arabidopsis accessions AT3G57240, AT3G57260, AT3G57270 and AT4G16260 have been reported to be induced by biotic stresses (Dong *et al.*, 1991; Doxey *et al.*, 2007). Five EGases from tobacco (accession numbers CAA38324, AAA34079, AAD33880, AAA34078 and AAA34103.1) and two from soybean (accession numbers AAA33946 and AAR26001), reported as PR (Rose *et al.*, 2002; Takeuchi *et al.*, 1990), were included in the analysis.

The putative expression of grapevine EGases was determined by the presence of associated cDNA clones in the grapevine Genome Browser at Genoscope (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>) and by the analysis of publicly available RNA-Seq data (see *In silico* expression analysis section).

Alignments were performed with Muscle and curated with GBlocks. Phylogenetic trees were constructed using PhyML with LG model and 100 bootstrap repetitions. Analyses were performed at <http://www.phylogeny.fr> (Dereeper *et al.*, 2008). Alignments in Fig. 1b were performed with CLUSTALW and identities were highlighted using BoxShade at Swiss EMBnet (<http://www.ch.embnet.org/>).

Semi-quantitative RT-PCR

Plasmopara viticola-infected material for RT-PCR experiments was obtained by inoculating leaf discs as described by Peressotti *et al.* (2010). Leaf discs were prepared from the third and fourth leaf counting from the apex from *V. vinifera* seedlings. Discs were bulked and distributed in Petri dishes. Leaf discs were inoculated by spraying a solution of 50 000 sporangia/mL. Mock-inoculated material was obtained by replacing the *P. viticola* suspension with water. At each time point, 10 leaf discs were snap frozen in liquid nitrogen and conserved at -80°C. RNA extraction, first-strand cDNA synthesis and PCR amplification were performed as described previously (Santos-Rosa *et al.*, 2008). Amplifications consisted of 25 cycles of 20 s at 94°C, 20 s at 60°C and 60 s at 72°C, followed by a final extension step of 10 min at 72°C. PCR products were separated on 1% agarose gels in 1 × Tris-Acetate-EDTA (TAE) and stained using ethidium bromide. Primer sequences are listed in Table S3 (see Supporting Information).

In silico expression analysis

Sixty-five grapevine RNA-Seq raw data sequence files generated within the framework of seven different experiments (Da Silva *et al.*, 2013; Jones *et al.*, 2014; Perazzolli *et al.*, 2012; Ramos *et al.*, 2014; Sweetman *et al.*,

2012; Vannozzi *et al.*, 2012; Venturini *et al.*, 2013) were retrieved from the National Center for Biotechnology Information (NCBI) SRA public database (<http://www.ncbi.nlm.nih.gov/sra>). Data were formatted in the fastq format using the fastq-dump command from the SRA Toolkit package version 2.3.4 (<http://www.ncbi.nlm.nih.gov/books/NBK158900>).

RNA-Seq reads were mapped against the V1 annotation of the grapevine line 40024 12x genome sequence, stored in the Grape Genome Database hosted at CRIBI. Alignments were performed using GSNAP version 2013-11-27 (Wu and Nacu, 2010) with the parameters: -B 4, -N 1, -n 3, -nofails and the quality protocol according to the experiment. These files were parsed to keep the best, unique and paired (if paired-end reads) alignments using an in-house-developed script.

The number of fragments aligned was counted using the command htseq-count from the HTSeq framework version 0.6.0 (Anders *et al.*, 2015) with the parameters: -m intersection-nonempty and -s no. FPKMs (fragments per kilobase of exon per million fragments mapped) were calculated for every gene using an in-house-developed script.

Gene cloning, protein expression and purification

The three grapevine EGases were cloned and expressed as described previously (Belval *et al.*, 2015). Briefly, for transient expression in plants, sequences were PCR amplified using high-fidelity polymerases and cloned into binary plasmid pBIN61 using restriction endonucleases. For protein expression, sequences were amplified from binary clones, cloned into suitable plasmid vectors (pHGWA, Busso *et al.*, 2005) by recombination using BP clonase, and transformed into *E. coli* Arctic Express. The integrity of genes was verified by sequencing. The primers are listed in Table S3.

For bacterial expression of proteins, overnight culture (2 mL) was transferred into 200 mL of Lysogeny broth (LB) containing ampicillin (100 µg/mL) and chloramphenicol (50 µg/mL), and was grown for 3 h at 28°C. It was placed at 10°C for 1 h and protein production was induced by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 24 h at 10°C. Bacteria were collected by centrifugation (6000 g, 10 min), washed with cold phosphate-buffered saline (PBS), centrifuged again and suspended in 1 : 100 culture volume of PBS with protease inhibitors. Bacteria were then disrupted using a Branson sonifier 250 (Emerson, St Louis, MO, USA) until clarification, typically 3 × 30 s. Cell fragments were pelleted by a 10-min centrifugation at 12 000 g. Supernatant was collected, aliquoted and frozen at -20°C.

Enzymes were purified using Protino Ni-TED columns according to the manufacturer's instructions. To eliminate Cpn60 chaperone (GroEL) contamination, a supplementary wash after sample loading was performed according to Belval *et al.* (2015) for EGase1 and EGase3. Briefly, resin was washed with 5 mL of 2 M urea/20 mM Tris, pH 6.8. This urea wash was not necessary for EGase2 purification. After washing with the kit buffer (Lysis-Equilibration-Wash, LEW, 1×), enzymes were eluted with elution buffer in fractions of 250 µL. Protein concentration was determined by measuring the absorbance at 280 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Enzyme-containing fractions were pooled and dialysed/concentrated using Amicon Ultra 10k devices (Millipore, Molsheim, France), according to the manufacturer's instructions, against 50 mM NaCl/20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes), pH 7, containing EDTA-free protease

inhibitors. Protein concentration was measured as described above. Blank was performed with buffer containing protease inhibitor.

Protein electrophoresis

SDS-PAGE (12% acrylamide) was performed according to Laemmli (1970). Gels were stained using colloidal Coomassie blue (Neuhoff *et al.*, 1988).

In vitro enzymatic assays

Enzyme activities were determined by measuring the increase in reducing sugars released in a solution of laminarin in 0.1 M of sodium acetate or MOPS buffer, according to the methods of Karvonen and Somersalo (1952) and Somogyi (1952). The katal is defined as the enzyme activity catalysing the formation of 1 mole of glucose equivalent per second.

To determine the effect of pH on enzymatic activity, the specific activity of each EGase was measured in the pH range 3–7.5 in pH 0.5 increments, using sodium acetate buffer (0.1 M) for pH 3–6.5 and MOPS buffer for pH 6.5–7.5, keeping the temperature constant at 37°C.

To assess the effect of temperature, specific activities of EGases were measured at temperatures ranging from 0–4°C up to 50°C for EGase1 and EGase2, and 0–4°C up to 70°C for EGase3, by 10°C increments, in 0.1 M sodium acetate buffer, pH 5.4. The relative activities were then expressed as the percentage of the maximum specific activity. Three biological repeats were performed for each pH and temperature.

Measurements of kinetic parameters were performed at 37°C in 0.1 M sodium acetate buffer (pH 5.4) containing various concentrations of laminarin (0–2 mg/mL; 0–0.34 mM) for 5 min. K_M (μM) and k_{cat} (s^{-1}) were calculated using non-linear regression analysis with IgorPro (WaveMetrics, Inc., Lake Oswego, OR, USA). The enzyme quantity in the assays was varied between 1×10^{-5} and 8×10^{-5} μM .

Substrate specificity

The specific activities of the three enzymes were measured with laminarin and also with several substrates solubilized in 0.1 M sodium acetate, pH 5.4, in the conditions described in the previous section: dextran from *Leuconostoc* [linear $\alpha(1,6)$ -linked glucose units and $\alpha(1,3)$ -linked branches], carboxymethyl cellulose (β -1,4-D-glucopyranose polymer of cellulose), β -D-glucan from barley (a polymer of β -D-glucose with 70% of glucose residues 1,4-linked and 30% 1,3-linked; Kim *et al.*, 2011) and xylan (a polysaccharide composed of 1,4-xylose).

In planta glucanase activity assays

Transient expression of EGases in *N. benthamiana* leaves was performed by agroinfiltration. *Agrobacterium* containing the binary constructs was grown for 2 days at 28°C in 5 mL of medium containing 50 $\mu\text{g}/\text{mL}$ kanamycin and 2.5 $\mu\text{g}/\text{mL}$ tetracycline. After centrifugation, bacteria were resuspended in a solution containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mM MgCl_2 and 150 μM acetosyringone, incubated at room temperature for 2–3 h, and infiltrated at an optical density at 600 nm (OD_{600}) of 0.2 using a needleless syringe, as described in Scofield *et al.* (1996).

Two leaves of 4-week-old *N. benthamiana* seedlings were infiltrated with each EGase. Leaves were sampled at 2 days post-infiltration and pro-

tein crude extracts were prepared by grinding the leaves in extraction buffer (0.1 M sodium acetate, 14 mM β -mercaptoethanol, 1% polyvinylpyrrolidone-40, pH 5.2) using 6 mL of buffer per 1 g of fresh leaf. Glucanase activity was measured using laminarin as substrate (Derckel *et al.*, 1998).

Functional assays on downy mildew

Plasmopara viticola strain Resdur was collected from *V. vinifera* Chardonnay in the experimental field of INRA in Colmar (France) and maintained on seedlings of *V. vinifera* cultivar Muscat Ottonel grown at 21°C and 100% relative humidity. Five days after inoculation, sporangia were harvested from infected leaves by shaking in sterile distilled water and the suspension was diluted to 20×10^3 sporangia/mL. Aliquots of 1 mL of this suspension were placed in wells on 24-well plates and incubated for 2–3 h at 21°C to obtain germinated spores. Three biological repetitions were performed. In each experiment, a microscopic field ($640 \times 640 \mu\text{m}^2$ for two experiments and $1280 \times 1280 \mu\text{m}^2$ for one experiment) containing zoospores was selected to perform time-lapse observation. Then, 3 μL of a 1% FDA solution were added to each well to reveal live zoospores (Widholm, 1972). Imaging was started 2 min after FDA addition ($t = 0$ min). EGases were added ($t = 1$ min) and time-lapse experiments were performed for 12 min at 1-min intervals between frames. Samples in which the enzymes were replaced by buffer were used as negative controls. Zoospores were then manually counted on photographs at $t = 0$ min and $t = 12$ min in visible and fluorescent light. Fluorescent light reveals FDA fluorescence and indicates live zoospores, whereas visible light allows the counting of structurally intact zoospores. Counting data are presented in Table S2.

Observations were made using a confocal laser scanning microscope (Zeiss LSM510, Zeiss, Oberkochen, Germany) with an EC Plan-Neofluar (10X/0.30 M27) objective lens under multitrack mode. Excitation/emission wavelengths were 488 nm/505–550 nm for FDA-stained germinated spores. Images were analysed using ImageJ (Schneider *et al.*, 2012) and figures were mounted using the plugin FigureJ (Mutterer and Zinck, 2013).

Statistical analysis was performed using R version 2.13.1 (R Core Team (2013)).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Expression of grapevine endoglucanases on spraying with water.

Table S1 RNA-Seq data from the literature summarizing the mRNA expression of grapevine endo-β-1,3-glucanases (EGases).

Table S2 Raw data for spore counting used to prepare Fig. 6b,c.

Table S3 Primers used in this study.