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Expression of disease resistance in genetically modified grapevines correlates with the contents of viral sequences in the T-DNA and global genome methylation

Daniela Dal Bosco · Iraci Sinski · Patrícia S. Ritschel · Umberto A. Camargo · Thor V. M. Fajardo · Ricardo Harakava · Vera Quecini

Abstract Increased tolerance to pathogens is an important goal in conventional and biotechnology-assisted grapevine breeding programs worldwide. Fungal and viral pathogens cause direct losses in berry production, but also affect the quality of the final products. Precision breeding strategies allow the introduction of resistance characters in elite cultivars, although the factors determining the plant's overall performance are not fully characterized. Grapevine plants expressing defense proteins, from fungal or plant origins, or of the coat protein gene of grapevine leafroll-associated virus 3 (GLRaV-3) were generated by *Agrobacterium*-mediated transformation of somatic embryos and shoot apical meristems. The responses of the transformed lines to pathogen challenges were investigated by biochemical, phytopathological and molecular methods. The expression of a *Metarhizium anisopliae* chitinase gene delayed pathogenesis and disease progression against the necrotrophic pathogen *Botrytis cinerea*. Modified lines expressing a *Solanum nigrum* osmotin-like protein also exhibited slower disease progression, but to a smaller extent. Grapevine lines carrying two hairpin-inducing constructs had lower GLRaV-3 titers when challenged by grafting, although disease symptoms and viral multiplication were detected. The levels of global genome methylation were determined for the genetically engineered lines, and correlation analyses demonstrated the association between higher levels of methylated DNA and larger portions of virus-derived sequences. Resistance expression was also negatively correlated with the contents of introduced viral sequences and genome methylation, indicating that the effectiveness of resistance strategies employing sequences of viral origin is subject to epigenetic regulation in grapevine.

Keywords Chitinase · Epigenetics · Fungus · Grapevine leafroll-associated virus 3 · Pathogenesis related protein 5 · *Vitis*

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Introduction

Grapevine diseases caused by viruses and fungi impose severe economic losses, not only due to their direct effect on production and yield, but also by modifications of berry metabolism, which result in lower contents of soluble solids and increased acidity (Burger et al. 2017; Gadoury et al. 2007). Moreover, pathogen induced modifications in the fruit specialized metabolism are carried over to the final products, compromising their quality and fitness for consumption (Gadoury et al. 2007). Chemical control remains a major recommendation in global protection strategies against fungal diseases, whereas the combat of viral pathogens relies on prophylactic measures, such as the eradication of symptomatic plants, and reintroduction of virus-free certified material.

Genetic engineering is particularly attractive to improve plant tolerance and resistance to pathogens in perennial species, where conventional breeding is challenging due to severe inbreeding depression, long lifecycle, and lack of knowledge on resistance genes or genetic architecture of the trait (Gray et al. 2014). Moreover, worldwide viticulture largely relies on a restricted number of *Vitis vinifera* elite cultivars, and is reluctant to accept novel and hybrid cultivars, bred for pathogen resistance or tolerance purposes. Genetic engineering for disease resistance has been extensively used in grapevine (review in Gray et al. 2014), with various degrees of success. For fungal and bacterial diseases, the design of genetically engineered plants expressing proteins with antimicrobial activity, either from plants or other organisms, has been one of the most common strategies used (Breen et al. 2015). The coding sequences of pathogenesis-related (PR) proteins from wild *Vitis* species or other sexually incompatible plants and microorganisms were used to produce transgenic grapevines aiming fungus resistance (Gray et al. 2014). In contrast, virus resistance is frequently sought after by the introduction of viral genomic sequences into the plant host genome (Maliogka et al. 2015). Sequences derived from virus coat protein (CP) have been widely used in grapevine to induce resistance (Gambino et al. 2005, 2010; Jardak-Jamoussi et al. 2009; Mauro et al. 1995; Valat et al. 2006; Vigne et al. 2004).

In plants, the introduction and expression of foreign sequences and the presence of RNA pathogens has led to the discovery of various homology-dependent gene-silencing mechanisms associated to changes in DNA methylation (Hewezi et al. 2017; Matzke et al. 2015). Three context of DNA methylation have been described in plants; CG, CHG, and CHH (where H is A, C or T), requiring distinct mechanisms for their establishment and maintenance, and showing different effects on DNA sequences (Matzke et al. 2015). Frequently, gene expression is shut down in genome regions methylated for all three contexts, and the repression can sometimes be extended to neighboring locations (Hewezi et al. 2017; Matzke et al. 2015). Global genome methylation has been associated with gene expression changes in developmental control, evolutionary processes, and responses to environmental conditions in plants (Lewsey et al. 2016; Matzke et al. 2015). In grapevine, distinct local levels of methylation were associated to post-transcriptional gene silencing (PTGS) of viral sequences (Gambino et al. 2010) and silencing of virus-derived sequences could be induced by artificial micro RNAs (Jelly et al. 2012). The influence of exogenous sequence contexts on genome methylation and transgene expression in grapevine remains elusive.

In the this study, we have investigated the responses of genetically engineered grapevines to fungal and viral pathogens using plants obtained from distinct transformation methods, carrying exogenous sequences from different origins, and devised to induce resistance by distinct mechanisms. Moreover, multivariated and correlation analyses were performed to provide further insight on the effect of the molecular features of the transgenic plants on the expression of the resistance mechanisms in vivo.

Materials and methods

Plant material

Grapevine cultivars were obtained from the Germplasm Bank at Embrapa, and include a seedless cultivar, BRS Clara (Camargo et al. 2003) and the wine cultivar Chardonnay. 'BRS Clara' is a genotype with more than 85% of *V. vinifera* genome and indistinguishable responses to *B. cinerea* and grapevine leafroll-associated virus 3 (GLRaV-3) (Camargo et al. 2003). Accessions were provided as woody cuttings containing flower or vegetative buds, for somatic embryogenesis and in vitro plant propagation,

respectively. Genetic transformation of 'Chardonnay' and 'BRS Clara' employed somatic embryos (SE) and shoot apical meristems (SAM) as explants (Fig. 1, Fig. S1). Timeline for explant production, genetic transformation and plant analyses are schematically represented in Supplementary Fig. 1 (Fig. S1).

Genetic transformation binary vectors

The cDNA sequences of proteins with antimicrobial activity, namely, a chitinase from *Metarhizium anisopliae* (*MaCHIT1*) (GenBank accession AF027498) (Bogo et al. 1998) and an osmotin-like from *Solanum nigrum* (*SnOLP*) (GenBank accession AF450276) were excised from pMOG463 (Mogen, The Netherlands) and pCambia1390 (Cambia, Australia) by digestion with *Eco*RV/*Bam*HI and *Hind*III/*Eco*RI, respectively. The resulting fragments of 1.7 kb and 752 bp were gel purified with PureLink™ Quick Gel Extraction Kit (ThermoFischer Scientific, USA) and ligated to pCambia1304 at the multicloning site.

For cloning of *MaCHIT1*, pCambia1304 was digested with *Eco*RI, the 5′ overhangs filled with DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs Inc., USA) according to the manufacturer's instructions and, subsequently, digested with *Bam*HI. The transformation vector was labeled pMaCHIT1. For cloning of the *SnOLP* sequence, the 5′ overhangs of the digested fragment were blunted as described, purified by chloroform wash followed by ethanol precipitation and digested with *Eco*RI. The processed *SnOLP* fragment was ligated to pCambia1304 digested with *Eco*RI, resulting in transformation vector pSnOLP.

Two hairpin RNA constructs employing the 3′ or 5′ ends of the gene coding for the capsid protein (CP) of a Brazilian isolate of Grapevine leafroll-associated virus 3 (GLRaV-3) (GenBank accession AY753208) (Fajardo et al. 2007) were used. The fragments were amplified by polymerase chain reaction (PCR) using primers containing *Pst*I/*Nco*I and *Sac*I/*Kpn*I restriction sites. The oligonucleotides used in the current work are described in Supplementary Table 1 (Table S1). The sense and antisense fragments were stepwise cloned at the *Nco*I/*Kpn*I and *Pst*I/*Sac*I restriction sites of a vector containing the intron (426 bp) of a citrus *CHITINASE* (*CHI*) gene (GenBank accession AJPS01004869.1 from 32,028 to 33,224) as spacer to form the hairpin structure. The fragments containing 'senseCP-intron-antisenseCP' for the 5′ and 3′ extremities of the viral gene were removed by *Eco*RI/*Hind*III double-digestion and cloned independently to pCambia1304. Cloning of the fragments was determined by restriction analyses, and one clone was selected for each gene end (pGLR3-3′ and pGLR3-5′).

The structure and integrity of the sequences in the T-DNA in the binary vectors pMaCHIT1, pSnOLP, pGLR3-3′ and pGLR3-5′ were confirmed by sequencing and are schematically represented in Fig. 2. Purified binary vectors were employed to transform electrocompetent *Agrobacterium tumefaciens* EHA105.

Grapevine genetic transformation

Shoot apical meristems were transformed as described by Dutt et al. (2007) with the following modifications: the increase of explant mass was initiated with ten freshly excised shoots tips of approximately 1 cm in length, cultivated in 25 mL liquid C2D4B for 4 weeks under diffuse light with mild shaking (100 rpm) at 23 °C, and the blot dried explants were immediately used in transformation experiments, without the solid medium cultivation step (Fig. 1a, b). The explants were wounded by nicking with a fine pointed scalpel blade during the blotting step. After cocultivation, the explants were transferred to plates containing regeneration medium, consisting of MS (Murashige and Skoog 1962) basal salts, B5 vitamins (Gamborg et al. 1968), 30 g L^{-1} sucrose, 9.0 µM 6-benzylaminopurine (BAP), 0.1 µM naphthalene acetic acid (NAA), 200 mg L^{-1} carbenicillin, 200 mg L^{-1} cefotaxime, 25 mg L^{-1} hygromycin and solidified with 6.5 g L^{-1} agar. Medium pH was adjusted to 5.8 prior to autoclaving.

Somatic embryos were transformed as described by Li et al. (2006), except that embryos were precultured on fresh GS1CA medium (Iocco et al. 2001) for 6 days before transformation, and after cocultivation, the explants were washed in liquid GS1CA supplemented with 200 mg L^{-1} carbenicillin and 200 mg L^{-1} cefotaxime, before transfer to regeneration medium, consisting of GS1CA supplemented with 200 mg L^{-1} carbenicillin, 200 mg L^{-1} cefotaxime and 25 mg L^{-1} hygromycin, for 10 days, in the dark at 23 ± 2 °C (Fig. 1a, b). Individualized embryos able to form roots in hygromycin containing media
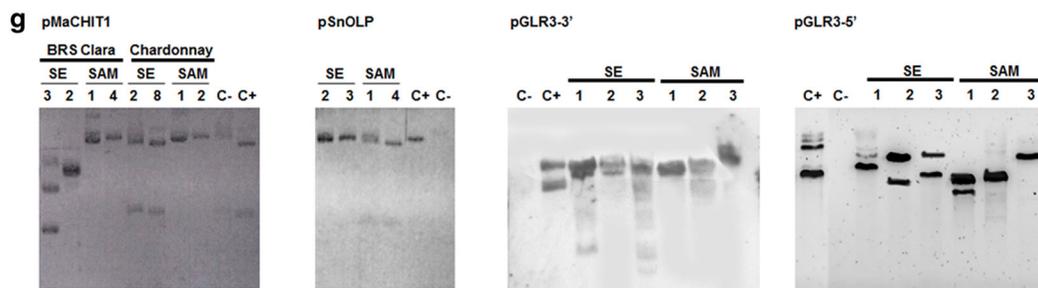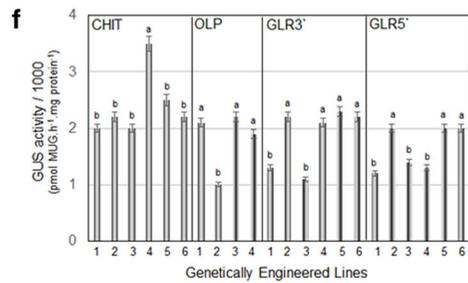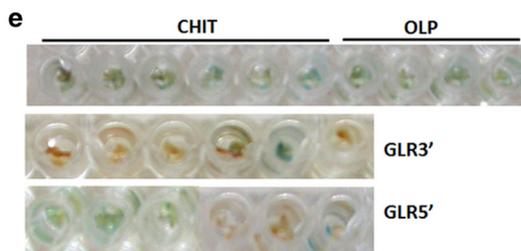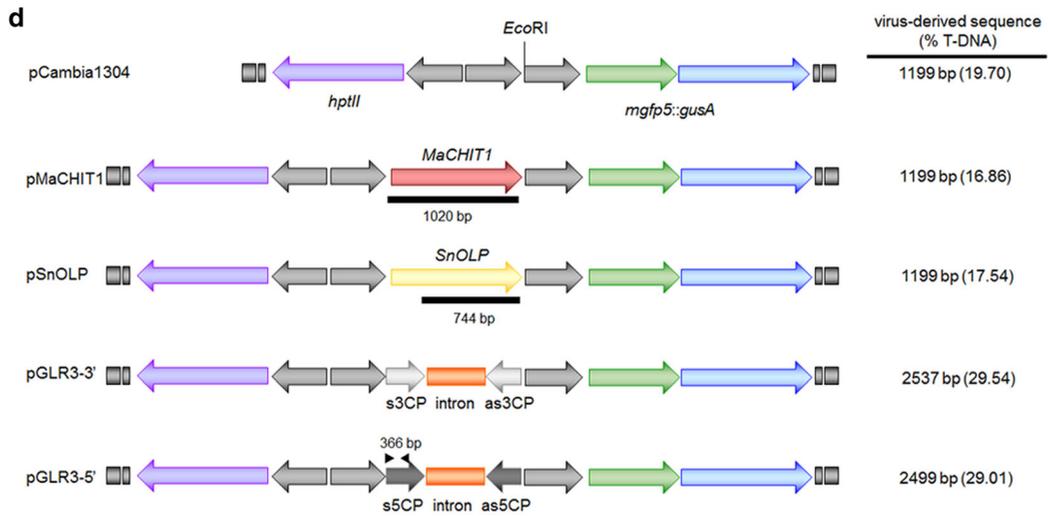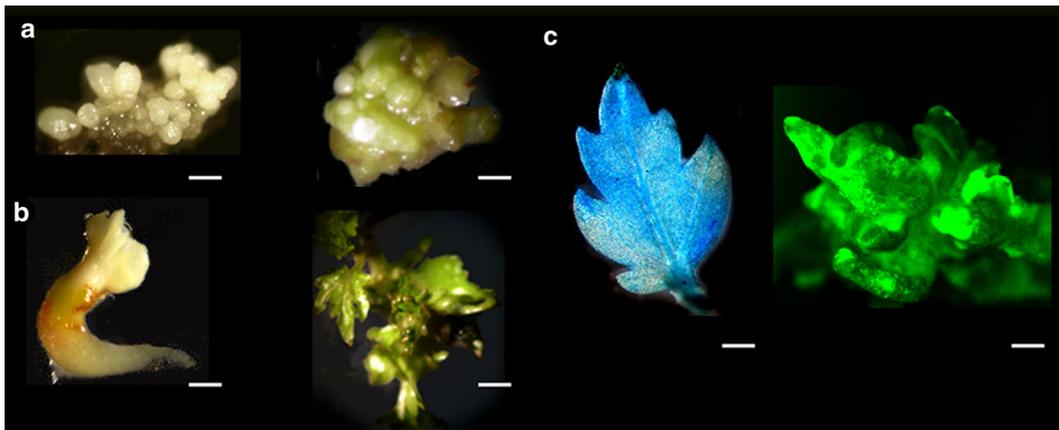
◄ **Fig. 1** Grapevine genetic engineering. Somatic embryos (SE) (left hand panel) and shoot apical meristem (SAM) (right hand panel) explants (**a**), regeneration (**b**) and GUS and GFP reporter gene expression in regenerated, rooted plantlets (**c**). Scale bar corresponds to 1 cm. Schematic representation of T-DNA regions of the vectors used to transform grapevine with the percentage of virus-derived sequence (**d**). Arrows represent 5′–3′ sequenced direction in the vector. Plant selection marker *HYGROMYCIN PHOSPHOTRANSFERASE* (*HPTII*) is represented in purple, reporter translational fusion *mgfp5::gusA* is shown in green and blue. Regulatory sequences CaMV35S promoter and *NOPALINE SYNTHASE* (NOS) polyadenylation signal, along with T-DNA borders are shown in gray, as arrows and bars, respectively. Hairpin-inducing viral sequences are represented as arrows for 3′ or 5′ coat protein (3CP or 5CP) coding sequences in sense (s) and antisense (as) orientation. Citrus *CHITINASE* intron is represented in orange. Position of a single cutter restriction enzyme (*Eco*RI) in the multicloning site of the transformation vectors is represented. DNA and RNA hybridization probes correspond to the T-DNA for all vectors, and to underlined fragments in pMaCHIT1 and pSnOLP. The position of PCR primers in the 5′ hpGLR3 line is represented by arrowheads, and the size of the amplified fragment, in base pairs. **e** Histochemical analyses of GUS expression in regenerated plants. **f** Fluorometric analyses of GUS activity in regenerated lines. Letters represent Tukey's HSD at $p < 0.05$ (one-way ANOVA). **g** Hybridization of *Eco*RI digested genomic DNA from transformed grapevine lines with T-DNA probes from the vectors used for genetic transformation. Negative control (C−) correspond to DNA from wild-type, non-engineered plants, and positive control (C+) to linear plasmid DNA (10 ng). (Color figure online)

(Fig. 1a, b) were checked for the presence and expression of the transgene by PCR and β-glucuronidase assays, respectively (Fig. 1a–c)

Molecular characterization and expression analyses

DNA hybridization analyses were used to confirm the stable insertion of the transgenes in the host genomes. Briefly, 15 μg of genomic DNA, extracted as described by Lodhi et al. (1994), were completely digested with *Eco*RI, which cuts once in the T-DNA, resolved on 1X TAE 0.7% (w/v) agarose gel and blotted to Amersham Hybond N$^+$ membranes (GE Healthcare and Lifesciences, USA). Sequences between the T-DNA borders were gel purified as probes (Fig. 1d) and labelled with alkaline phosphatase using AlkPhos Direct Labeling and Detection™ (GE Healthcare and Lifesciences, USA). Membranes were prehybridized for 60 min at 55 °C with gentle shaking (60 rpm). Subsequently, 10 ng of

labelled probe were added to the prehybridization solution and the membranes hybridized for 16 h at 55 °C. Blots were washed according to the manufacturer's instructions, and the CDP-Star™ reagent (GE Healthcare and Lifesciences, USA) was used to generate the chemiluminescent signal, detected on Amersham Hyperfilm ECL (GE Healthcare and Lifesciences, USA).

The transcription of the genes coding for exogenous proteins with antimicrobial activity was evaluated by RNA hybridization. A total of 12 μg of total RNA, extracted as described by Gambino et al. (2008), were resolved on 1X MOPS 0.7% (w/v) agarose denaturing gels. Nucleic acid transfer to the membrane, probe labelling and chemiluminescence detection were performed as described, except that the probes consisted of gel purified *MaCHIT1* and *SnOLP* fragments, instead of the whole T-DNAs as used for DNA hybridization (Fig. 1d). Reporter gene expression was determined by histochemical and fluorometric analyses for *gus A*, and fluorescent microscopy at 395 nm, for *egfp5*. Reporter expression levels are represented as scale, ranging from low (+) to high (+++) expression levels for *gusA* and *egfp5*. Expression levels were consistent for the reporter genes in all investigated lines.

Disease response analyses

Independently transformed, non-chimeric lines were randomly chosen for analyses, namely: two 'BRS Clara' and one 'Chardonnay' lines carrying pMaCHIT1, two 'Chardonnay' and one 'BRS Clara' carrying pSnOLP, six 'Chardonnay' lines carrying pGLR3-3′ and six, pGLR3-5′; along with pCambia 1304 transformed and wild type 'BRS Clara' and 'Chardonnay' controls. The chosen lines were in vitro propagated and, at least five plants of each line, were employed on pathogen challenging. The investigated lines were obtained from both regeneration systems, namely, shoot apical meristem (SAM) and somatic embryo (SE) transformation (Table 1, Fig. S2).

Botrytis cinerea infection assay

A local isolate of *B. cinerea* from the fungal pathogen collection at Embrapa was used. Monosporic cultures were plated on potato-dextrose-agar (PDA) medium in Petri dishes at 25 °C in the dark. Prior to inoculation,

Fig. 2 Disease response assays for chitinase (**a–d**), osmotin-like protein (**e–g**) and GLRaV-3 (**h–j**). Six representative chitinase and osmotin-like expressing lines were chosen for pathogen challenge. The lines are identified as CHIT1, CHIT2, CHIT3, OLP1, OLP2 and OLP3 in Table 1. Leafroll disease evaluation in response to grafting with GLRaV-3 was carried out for 12 lines, described in Table 1. **a** In vitro assay of chitinase activity. Letters represent Tukey's HSD at $p < 0.05$ (one-way ANOVA). **b** RNA hybridization employing a gene-specific probe. **c** Disease progression evaluated by the percentage of damaged leaf area after infection. **d** Light microscopy analyses of fungal structures in infected leaf discs at day 9. **e** Disease progression evaluated by the percentage of damaged leaf area after infection. **f** RNA hybridization employing a gene-specific probe. **g** Light microscopy analyses of fungal structures in infected leaf discs at day 5. **h** Frequency and intensity of leafroll symptom in hpGLR3 lines. **i** RT-PCR amplification of a GLRaV-3 specific fragment in symptomatic engineered plants. **j** Heatmap representation of GLRaV-3 coat protein levels detected by ELISA in genetic engineered and wild-type control plants. (Color figure online)
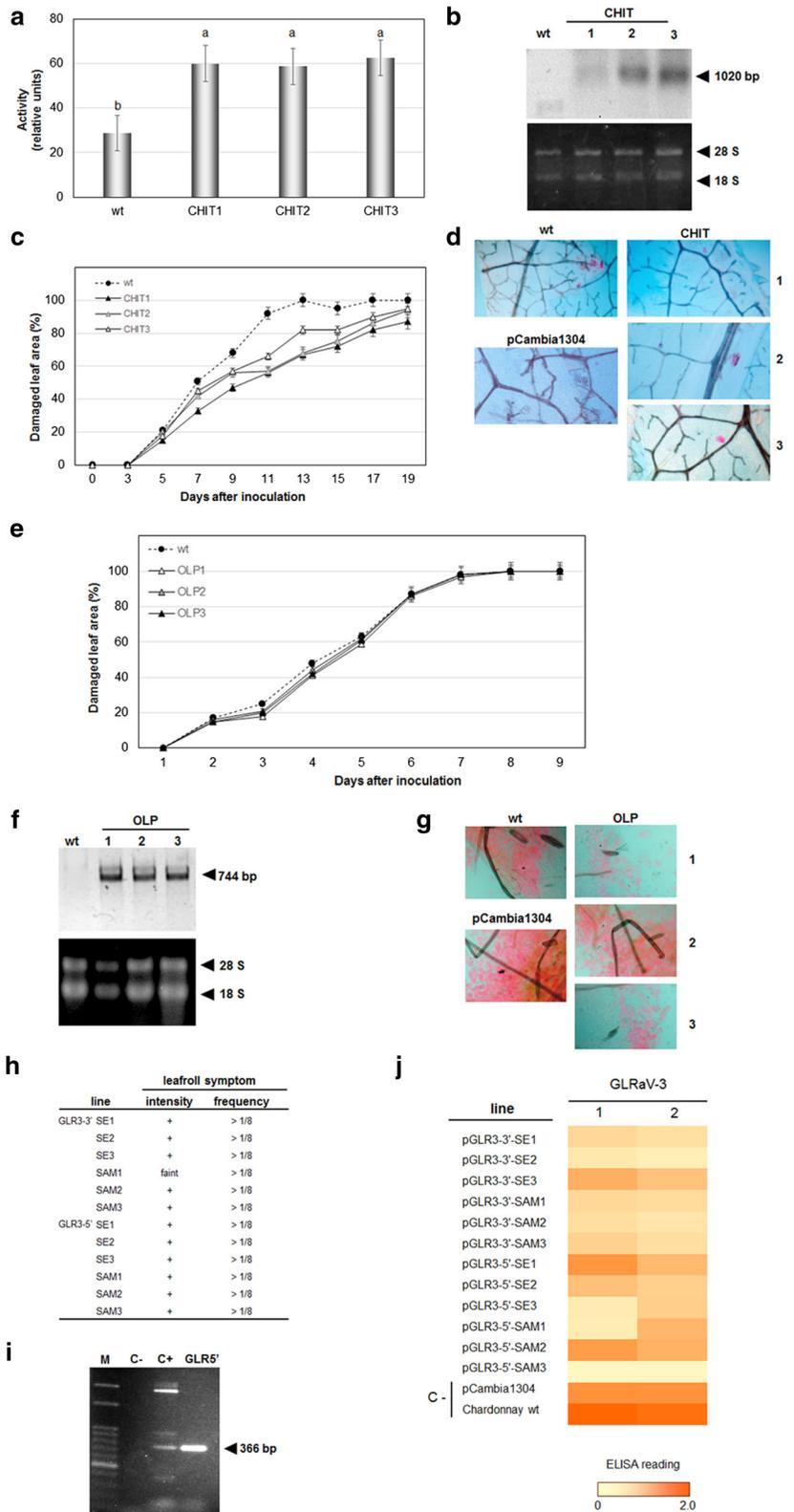
Table 1 Characterization of the genetically engineering and control grapevine lines used in pathogen challenge assays

Line	Species	Cultivar	Explant	Gene of interest	T DNA copy number	Expression level	
						Gene of interest	Reporter
CHIT1	*Vitis* hybrid	BRS Clara	SAM	*MaCHIT1*	1	++	++
CHIT2	*Vitis vinifera*	Chardonnay	SE	*MaCHIT1*	2	+++	++
CHIT3	*Vitis* hybrid	BRS Clara	SE	*MaCHIT1*	3	+++	+++
CHIT4	*Vitis* hybrid	BRS Clara	SAM	*MaCHIT1*	1	++	++
CHIT5	*V. vinifera*	Chardonnay	SAM	*MaCHIT1*	1	++	++
CHIT6	*Vitis* hybrid	BRS Clara	SE	*MaCHIT1*	1	+	++
CHIT7	*V. vinifera*	Chardonnay	SAM	*MaCHIT1*	1	+++	++
CHIT8	*V. vinifera*	Chardonnay	SE	*MaCHIT1*	2	++	+
OLP1	*Vitis vinifera*	Chardonnay	SAM	*SnOLP*	2	+++	++
OLP2	*Vitis* hybrid	BRS Clara	SE	*SnOLP*	1	+++	+
OLP3	*Vitis vinifera*	Chardonnay	SE	*SnOLP*	1	+++	++
OLP4	*Vitis* hybrid	BRS Clara	SAM	*SnOLP*	2	++	++
GLR3′-1	*V. vinifera*	Chardonnay	SAM	hpGLR3′	2	nd	+
GLR3′-2	*V. vinifera*	Chardonnay	SAM	hpGLR3′	1	nd	++
GLR3′-3	*V. vinifera*	Chardonnay	SAM	hpGLR3′	2	nd	++
GLR3′-4	*V. vinifera*	Chardonnay	SE	hpGLR3′	1	nd	++
GLR3′-5	*V. vinifera*	Chardonnay	SE	hpGLR3′	1	nd	+
GLR3′-6	*V. vinifera*	Chardonnay	SE	hpGLR3′	1	nd	++
GLR5′-1	*V. vinifera*	Chardonnay	SAM	hpGLR5′	2	nd	+
GLR5′-2	*V. vinifera*	Chardonnay	SAM	hpGLR5′	2	nd	+
GLR5′-3	*V. vinifera*	Chardonnay	SAM	hpGLR5′	2	nd	++
GLR5′-4	*V. vinifera*	Chardonnay	SE	hpGLR5′	2	nd	++
GLR5′-5	*V. vinifera*	Chardonnay	SE	hpGLR5′	1	nd	++
GLR5′-6	*V. vinifera*	Chardonnay	SE	hpGLR5′	1	nd	+++
p1304	*Vitis* hybrid	BRS Clara	SAM	na	2	na	++
p1304	*Vitis* hybrid	BRS Clara	SE	na	1	na	++
p1304	*V. vinifera*	Chardonnay	SAM	na	2	na	++
p1304	*V. vinifera*	Chardonnay	SE	na	1	na	+
wt	*Vitis* hybrid	BRS Clara	SAM	na	0	na	na
wt	*Vitis* hybrid	BRS Clara	SE	na	0	na	na
wt	*V. vinifera*	Chardonnay	SAM	na	0	na	na
wt	*V. vinifera*	Chardonnay	SE	na	0	na	na

The number of inserted T DNA copies was determined by DNA hybridization. Expression analyses were carried out using the original transformed line (T0), prior to plant propagation. Expression levels were determined by RNA hybridization for the genes of interest, and fluorometric assay for *GUSA*. Expression levels are represented as (+) low, intermediate (++) and (+++) high scale

CHIT, *Metarhizium anisopliae CHITINASE1*; GLR, 3′ or 5′ fragment of the coat protein (CP) gene from Grapevine leafroll-associated virus 3 (GLRaV-3); OLP, *Solanum nigrum OSMOTIN-LIKE1*; na, non-available; nd, not determined; p1304, binary vector pCambia1304; wt, wild type

isolated spores were transferred to fresh medium and incubated at 20 °C, under a 16 h photoperiod, until complete fungal growth (approximately 7 days). The adaxial surface of freshly detached leaves from in vitro plants (third fully expanded leaf from the top down) were inoculated by contact with agar slices (5 mm diameter) containing the grown fungus. Inoculated leaves were incubated as previously on magenta boxes

Fig. 3 Quantitative analyses of global genome methylation (ng) in CHIT, OLP and hp-GLR3 engineered grapevine lines. The amount of methylated DNA in plants carrying the empty vector (pCambia1304) and wild-type controls is shown in the insert. Wild type controls correspond to the average global methylation of 'BRS Clara' and 'Chardonnay' plants regenerated in vitro via somatic embryogenesis and direct organogenesis of shoot apical meristems. (Color figure online)
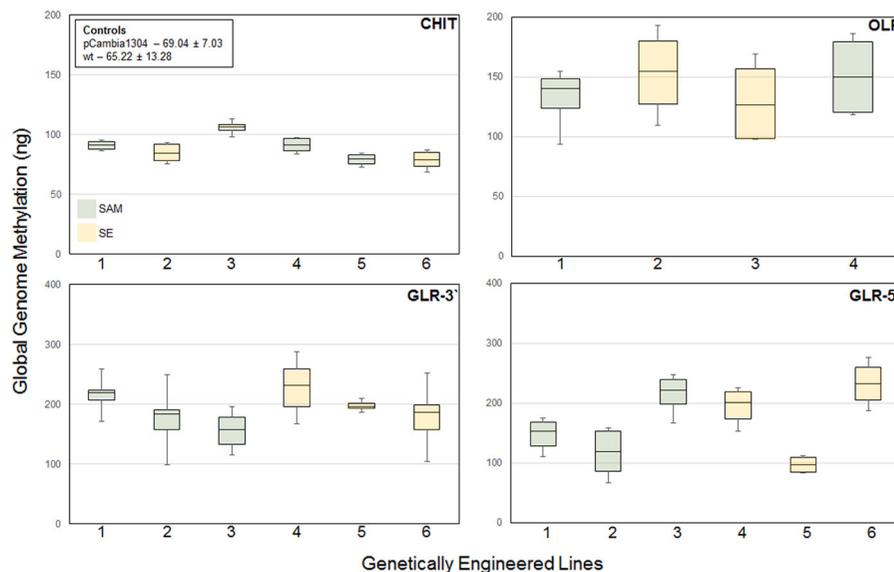
containing sterile moist filter paper, employing sterile rubber meshes to prevent direct contact with the wet filter paper. Negative controls consisted of agar slices without fungus. Five representative leaves from each treatment were digitally imaged daily after inoculation and lesions were measured using ImageJ 1.8 (Schneider et al. 2012). Plant tissue colonization by fungal structures was visualized by light microscopy in leaves cleared by chloral hydrate and stained by Schiff's reagent.

Chitinase activity assay

Total protein extracts were obtained from 100 mg of fresh plant tissue, finely ground in 1 mL of extraction buffer (100 mM acetate buffer; pH 5.0, 0.5 M NaCl, 1% (w/v) polyvinylpyrrolidone (PVP40), 0.1% Triton X-100, 2% (v/v) β-mercaptoethanol and 20 (v/v) % glycerol). The mixture was incubated at 25 °C for 3 h, centrifuged at 5000g for 5 min and the clear supernatant employed for protein quantification using the Bradford method. Protein extracts (150 μL) were added to the reaction mixture consisting of 0.1% colloidal chitin (300 μL) and 0.1 M phosphate buffer pH 7.0 (150 μL), incubated at 55 °C for 10 min and centrifuged at 4 °C at 10,000g for 5 min. The supernatant (200 μL) was added to 500 mL of deionized water and 1 mL of Schales reagent (0.5 M sodium carbonate and 0.5 g L^{-1} potassium ferricyanide in water), and boiled for 10 min. The absorbance of the

cooled mixture was determined spectrophotometrically at 420 nm.

Virus symptom and serological analyses

Lateral buds of approximately 1.2 cm in diameter were removed from 'Chardonnay' stock plants, positive for GLRaV-3 infection, determined by RT-PCR, and used as inoculum source. The buds were grafted to the stems of lignified plants carrying the hp-inducing construct, approximately 28 months after *ex vitro* acclimation. The responses to viral infection were evaluated by visual score of leaf roll symptoms and by two independent serological analyses, approximately 210 days after grafting. For virus titer determination, phloem samples were scraped from mature canes, and the crude extracts were diluted at 1:1.5 (w/v) to be used in Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) with polyclonal antisera against GLRaV-3 [IgG and conjugated antibody at 1:250 (v/v) from Agritest (Valenzano, Italy)].

Determination of global genome methylation

Global DNA methylation was quantified using Imprint® Methylated DNA Quantification Kit (Sigma Aldrich, USA) for triplicates of DNA isolation experiments from transgenic grapevine lines, methylated and blank controls, and duplicated absorbance readings according to the manufacturer's instructions.
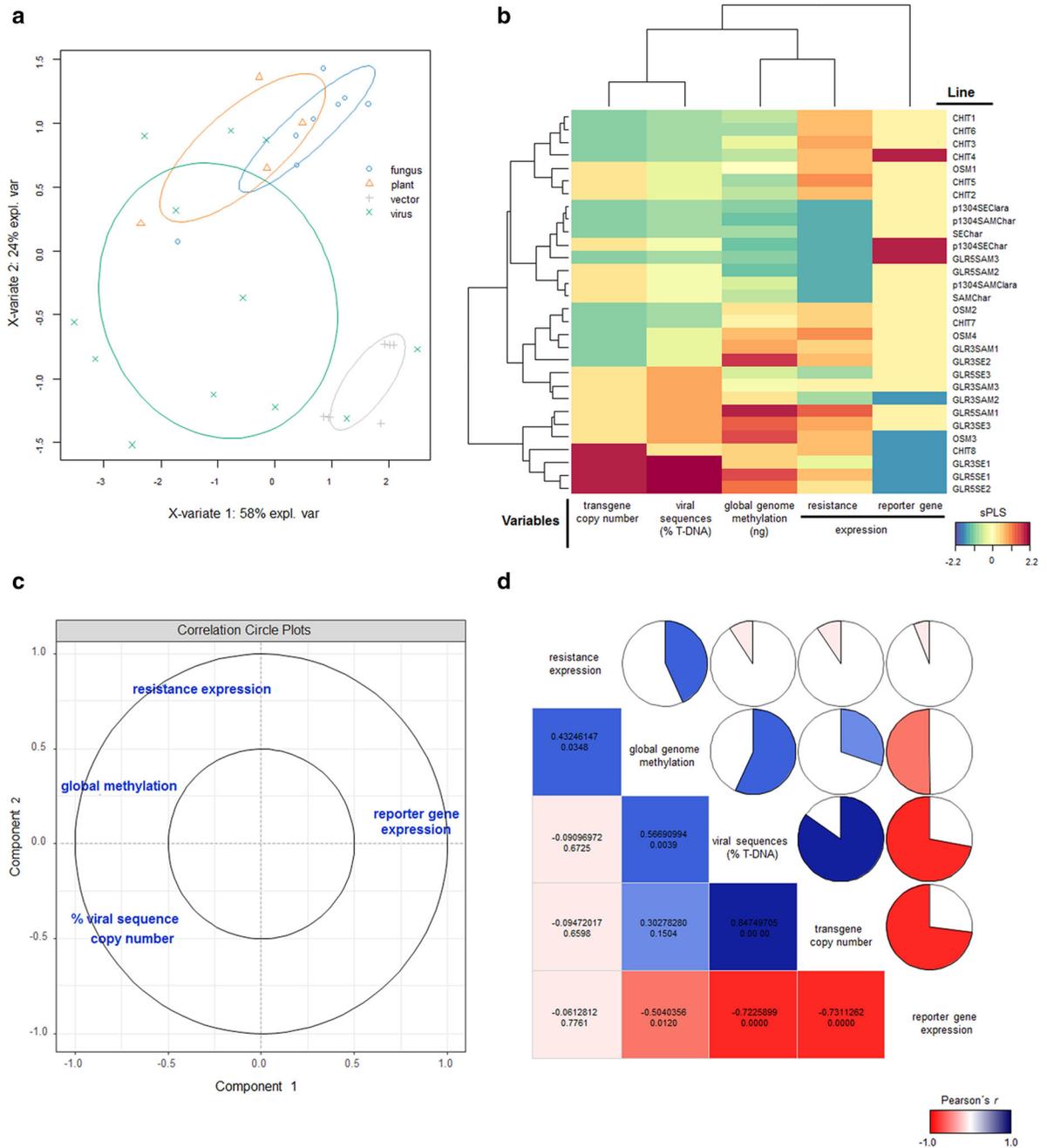
Fig. 4 Multivariate analyses of the transgene copy number, percentage of viral sequences, global genome methylation, and reporter gene and resistance expression in grapevine genetically engineered lines. **a** sPLS-DA plot of the individuals using the origin of the gene of interest as discriminant. Ellipses represent 95% confidence levels. **b** Clustered image map of the similarity matrix obtained by sPLS-DA results. Similarity is represented as heatmap, ranging from − 2.2 (blue) to 2.2 (red), and dendrograms derived from hierarchical clustering of the similarity results are represented for the genetically engineered lines (vertical) and variables (horizontal). **c** Correlation circle plot of the variables used in sPLS-DA analysis. **d** Heatmap and pie graph representation of the Pearson correlation between the investigated variables for the genetically engineered grapevine CHIT, OLP and hp-GLR3 lines. Pearson's r is given inside the squares along with its p value. (Color figure online)

DNA concentration was spectrophotometrically determined and adjusted to 500 ng. Contents of methylated DNA were determined by regression analysis of the standard curve generated by subtracting the absorbance readings at 450 nm (A_{450}) from the methylated ($A_{450}MC$) and blank ($A_{450}B$) controls versus the mass (ng) of methylated DNA for each point read. Data points for methylation quantification standard curve are presented in Supplementary Fig. 3 (Fig. S3).

Statistical analyses

Data were processed and analyzed in R (R Core Team 2015), using F statistics and Waller–Duncan k-ratio t test at $p < 0.05$. Regression analyses were done employing the fit function, and regression diagnosis was carried out by investigation of heteroscedasticity, normality, multicollinearity, non-linearity, and non-independence of errors, with the car package (Fox and Weisberg 2011). Multivariate analyses principal component analysis (PCA) and sparse partial least squares discriminant analysis (sPLS-DA) were performed and graphed using the mixOmics package (Lê Cao et al. 2016). Hierarchical clustering was done by simultaneously grouping rows and columns of the real-valued similarity matrix generated by sPLS-DA (Lê Cao et al. 2016). Pearson correlation analysis and its significance were calculated using Hmisc (Harrell Jr et al. 2017) and plotted employing the corrgram package (Wright 2017).

Results

Genetic engineering, transgene insertion and expression

Somatic embryos and shoot apical meristems were used as explants in *Agrobacterium*-mediated transformation to produce genetically engineered grapevine plants of the cultivars 'Chardonnay' and 'BRS Clara' (Fig. 1a, b). Embryos and shoots growing on selective hygromycin media and expressing the construct reporter genes (Fig. 1a–c) were recovered at approximately similar rates ($3.21\% \pm 0.38$ and $2.76\% \pm 0.25$, respectively). A small portion of SE from both cultivars ($0.74\% \pm 0.07$) developed abnormal leafy cotyledons, and did not produce viable plants. Chimeric regenerants, identified by reporter gene expression analyses, represented 1.17 and 1.09%

of the SAM- and SE-derived transformants, and were eliminated. External morphology, growth pattern, leaf color, and vigor of the rooted plants were identical to those of the wild type genotypes. Twenty-four genetically engineered lines carrying the genes of interest, along with the corresponding wild type and binary vector transformed controls were analyzed, and a selected subset, submitted to viral and fungal pathogen challenges (Table 1).

Histochemical detection of GUS activity was observed in leaf samples from all putative transgenic lines, as variable levels of diffuse staining after 24 h at 37 °C in the presence of glucuronide substrate (Fig. 1e). To quantify reporter gene expression, fluorometric readings of the reporter gene activity were determined. As observed for histochemical analyses, GUS activity evaluated by fluorometry was also variable for the transformed lines, ranging from 3740 to 950 pmol of MUG per h per mg of protein (Fig. 1f). Stable transgene insertion was confirmed by DNA hybridization analyses, and the estimated number of copies ranged from 1 to 3, employing DNA digested with a single cutter in the T-DNA and the T-DNA as probe (Fig. 1g, Table 1).

The genetically modified plants were further characterized by expression of the proteins with antimicrobial activity and biological challenges with a fungal pathogen by in vitro assays. The plants carrying virus RNA silencing constructs were propagated, gradually acclimated *ex vitro*, and challenged against GLRaV-3 by grafting.

Disease responses to *Botrytis cinerea*

Transgenic plants carrying CHIT and OLP constructs were maintained in vitro, by nodal segment propagation at 60-day intervals, and *Botrytis* pathogenesis was investigated by leaf disc assays for three representative lines of each construct (Table 1, Fig. 2).

RNA hybridization confirmed the transcription of the genes coding for proteins with antimicrobial activity in the tested transgenic lines (Fig. 2b, f). Chitinase activity was detected in wild type, nontransformed plants, although chitin degradation was approximately two times higher in lines carrying the CHIT construct (Fig. 2a). In contrast, the mRNA fragment of approximately 1 kb corresponding to *MaCHIT1* was not detected in wild type plants, although cross hybridization with an 850 bp fragment

remained at the employed stringency (Fig. 2b). Levels of *MaCHIT1* mRNA were higher in line 3, followed by line 2 (32.5% of line 3) and line 1 (17.5% of line 3), as inferred by densitometry analyses of the hybridization signal strength (Fig. 2b). Cross hybridization signal corresponded to 11.7% of the strongest signal (Fig. 2b). In pathogen challenged plants, chlorotic symptoms were visible on detached leaf discs 3 days after fungus inoculation, progressing to necrotic lesions at day 5 for wild type and genetically engineered CHIT2 and CHIT3 lines (Fig. 2c). Eleven days after inoculation, the affected leaf area was approximately 1.53 times smaller in CHIT lines (60% ± 3.06) than in the wild type (92% ± 4.12) (Fig. 2c), correspondingly the frequency of fungus-derived structures was higher in wild type and empty pCambia1304 lines than in all three investigated CHIT lines (Fig. 2d).

Disease symptom progression in OLP lines was not distinct from the wild type control (Fig. 2e). At early infection stages (day 2–5), the percentage of affected leaf area progressed slightly faster in wild type control leaves, although not significantly (Fig. 2e). Complete leaf necrosis occurred at day 9 for all lines, preventing further investigation (Fig. 2e). Accordingly, the frequency of fungal conidiophore and hyphae was high in all lines at day 9 (Fig. 2g), although RNA hybridization analysis confirmed the presence of transcripts corresponding to *SnOLP* in the investigated transgenic lines (Fig. 2f). Steady-state levels of *SnOLP* mRNA were similar in the three OLP lines (Fig. 2f).

Disease response to virus pathogens

Mature acclimated plants, carrying the hp-inducing construct, were grafted with lateral buds, positive for GLRaV-3, visually evaluated for typical leafroll symptoms, and molecularly characterized by PCR amplification of viral RNA sequences and CP antibody ELISA (Fig. 2h–j). For all transformed lines more than one, out of the eight inoculated clones, exhibited the leafroll phenotype, except for the line pGLR3-3′-SAM1, that displayed fainter symptoms (Fig. 2h). In agreement with visual observations, a GLRaV-3 specific fragment was successfully detected in pooled RNA samples from the genetically engineered lines (Fig. 2i).

Plants carrying hpRNA constructs exhibited lower virus titer than wild type and empty-vector containing plants; although detectable virus accumulation (CP levels) were present (Fig. 2j). For most lines, the levels of virus silencing tended to remain constant or slightly increase between the evaluations, except for lines pGLR3-5′-SE3 and pGLR3-5′-SAM1 (Fig. 2j). Average GLRaV-3 level of relative virus accumulation, corresponding to coat protein concentration, was approximately 3.2 and 2.4 times higher in wild type and empty vector transformed plants, respectively, in comparison to the levels found in hpRNA modified plants (Fig. 2j).

Constructs targeting the 5′ end of the virus gene were approximately 40% more effective to reduce the titers of virus coat protein, although the average copy number was also higher for the lines carrying the 5′ vector (m = 1.67 ± 0.52 and m = 1.34 ± 0.52, respectively) (Table 1, Fig. 2j). Moreover, the levels of virus silencing decreased in the second evaluation for two 5′ lines (Fig. 2j). Lines obtained from shoot apices had a higher average number of transgene copies (m = 1.83 ± 0.41), in comparison to those derived from somatic embryos (m = 1.17 ± 0.41), and exhibited approximately 20% lower levels of silencing of virus replication (Table 1, Fig. 2j).

Global genome methylation analyses

The global contents of methylated DNA in wild type and genetically engineered grapevine lines were determined using a commercial ELISA-based method (Fig. 3). Methylated control DNA, provided by the manufacturer, was used to calculate a standard curve, which had a correlation coefficient higher than 93% (Fig. S3). The lowest levels of global genome methylation were found in untransformed controls (65.22 ng ± 13.28) and in plants carrying the empty binary vector (69.04 ng ± 7.03) (Fig. 3). The different cultivars and explant types used in transformation did not significantly influence genome methylation contents (*p* values of 0.104 and 0.689, respectively). Although differences in the methylation levels from biological replicates are routinely up to 20%, as stated by the manufacturer, the amplitude of the readings was of 2.3 and 7.5% among CHIT and OLP lines, respectively (Fig. 3). In contrast, differences in methylation up to 47% among replicates were observed for lines carrying the hpRNA-inducing constructs (Fig. 3). Moreover, the magnitude of global genome methylation was higher for virus-derived

constructs (157.1 ng $\pm$ 58.2), with individual lines reaching more than 220 ng of methylated DNA, in comparison to the plants carrying genes of interest of fungal and plant origin (122.8 ng $\pm$ 41.5) (Fig. 3).

To gain further insight on the response of genetically engineered grapevines to pathogen challenges, the variables transgene copy number, percentage of viral sequences in the T-DNA, reporter and disease resistance expression, and global genome methylation were submitted to multivariate analyses. A large portion of the observed variance (84%) was explained by the first two principal components, although no clear association of the individuals to components was observed (Fig. S4). The variance/covariance structure of the data was better explained using linear combinations of the original variables in a sparse partial least square (sPLS) regression procedure using the origin of the gene of interest as discriminant (DA) (Fig. 4a). Hierarchical clustering of the sPLS-DA similarity matrix also demonstrated that lines carrying exogenous genes of plant and fungal origin exhibit distinct behavior in comparison to those of viral origin for the investigated variables (Fig. 4b). Reporter gene expression was the most divergent variable among the studied characteristics (Fig. 4c). Bivariate correlation analyses of the variables in the genetically engineered lines carrying resistance genes demonstrated a close association between the percentage of viral sequences in the T-DNA and the level of global genome methylation (Person's $r = 0.56690994$, p value < 0.0039) (Fig. 4d). The highest positive correlation was observed between transgene copy number and the percentage of viral sequences in the T-DNA (Person's $r = 0.84749705$, p value < 0.00), as expected (Fig. 4d). In contrast, the highest negative correlation was observed between the expression of the reporter gene and resistance ($r = -0.7311262$, p value < 0.00) (Fig. 4d). In fact, the expression of the reporter gene was negatively correlated with all other investigated parameters, namely, contents of viral sequences in the T-DNA, genome methylation, and resistance expression (Fig. 4d).

Discussion

Biotic stresses, including fungal and viral diseases, are considered a major challenge to sustainable grapevine production worldwide (Burger et al. 2017). Novel biotechnological approaches allow precise breeding interventions in elite cultivars, via many tools of genetic engineering and genome editing (review in Gray et al. 2014). However, several non-accounted for factors appear to affect the manifestation of the introduced character, especially after long-term pathogen exposure or under heterogeneous environmental conditions (Gambino et al. 2010; Li et al. 2015; Rubio et al. 2015). In the present study, we have investigated the in vitro and in vivo response of genetically engineered grapevines to pathogen challenges, employing modified lines carrying genes of plant, fungal and viral origins that were obtained via two distinct explant regeneration pathways, namely: somatic embryogenesis and direct organogenesis from shoot apices.

Ectopic expression of proteins with antimicrobial activity slows down disease evolution

Grapevine responses to *B. cinerea* initially employ structural defense mechanisms, consisting of pre-existent physical barriers, such as cuticle and trichomes (Wan et al. 2015). The initial responses are coupled with inducible pathways, leading to strengthening of the plant cell wall, secretion fungus cell wall degrading enzymes, and production of proteins involved in oxidative burst, or with antimicrobial properties (Haile et al. 2017). These responses were shown to restrict the growth of the invasive hyphae into the underlying plant tissues, which leads the pathogen to remain quiescent until favorable conditions occur and allow the evolution of pathogenesis (Haile et al. 2017). Changes in the plant developmental programs and cellular signals are hypothesized to trigger pathogen egression resuming the pathogenic process (Haile et al. 2017). The proteins ectopically expressed in the current study are associated to the initial stages of infection, namely: hydrolysis of the cell wall in several fungal structures (chitinase, in CHIT lines) (Bogo et al. 1998) and toxic effects to actively growing points (osmotin-like, in OLP lines) (Campos et al. 2008; Chowdhury et al. 2017).

The proteins *Ma*CHIT and *Sn*OLP were not capable of fully preventing fungal colonization of grapevine tissues, although they were demonstrated to be transcriptionally expressed and, in the case of CHIT, active. Detached leaf assays are considered drastic and may not reflect pathogenesis kinetics in intact plants

(Liu et al. 2007). In detached *Arabidopsis* leaves, pathogenesis evolution against hemibiotrophic *Colletotrichum* species was compromised even in the presence of the functional resistance locus *RCH1* (Liu et al. 2007). *Botrytis cinerea* pathogenesis relies on a combination of strategies to attack the plant cells, including the release of toxic metabolites, lytic enzymes, and microRNAs (Zhang et al. 2017). Thus, defense requires extensive transcriptional reprogramming and rarely results in complete symptom suppression (Zhang et al. 2017).

The rate of disease progression was reduced in most of the genetically engineered lines. In general, the ectopic expression of chitinase was more effective in delaying pathogenesis than of the osmotin-like protein. Fungal chitinases are classified to the glycosyl hydrolase (GH) 18 family and can be further separated into three groups and five classes (I–V), based on their domain architecture and sequence similarity (Langner and Göhre 2016). Although GH18 chitinases share a common catalytic mechanism, the enzymes are capable of endo and exoactivity, hydrolyzing sites within the polymer or from the termini (Langner and Göhre 2016). As shown previously, wild type grapevine plants also secrete native chitinases, such as the products of the genes *VvCHIT1a* (acidic class I chitinase) and *VvCHIT1b* (basic class I chitinase) (Chong et al. 2008; Robert et al. 2002). Due to its catalytic activity towards fungal cell walls, hyphal tips, and germ tubes chitinase genes from plant and fungal origins have been widely employed in genetically engineered plants to increase disease resistance. As shown in our work, the ectopic expression of microbial chitinase genes slowed down disease progression, and reduced fungal colonization in a wide range of other genetically engineered species, including tobacco, apple, potato and pea expressing *CHIT* genes from *Rhizopus oligosporus* (Terakawa et al. 1997), *Saccharomyces cerevisiae* (Carstens et al. 2003), *Trichoderma atroviride* (Bolar et al. 2001), *Streptomyces griseus* (Raham et al. 2008) or *Streptomyces olivaceoviridis* (Hassan et al. 2009). As observed in previous studies (Núñez de Cáceres González et al. 2015; Yamamoto et al. 2000), our results also demonstrated that higher levels of chitinase activity resulted in slower pathogen colonization.

Originally, osmotin was identified as a salt-induced protein in tobacco (*Nicotiana tabacum* L.) cells (Singh et al. 1989). With the increase of genomic data, osmotin and OLPs were demonstrated to be part of the widespread superfamily of thaumatin-like proteins (TLP) (Liu et al. 2010). In general, the antifungal features of TLPs are attributed to the variety of enzymatic activities displayed by these proteins against microorganism cell walls, including glucanase and other pore forming activities (Chowdhury et al. 2015; Liu et al. 2010). Endogenous and ectopic OLPs were also shown to participate in the defense against biotic factors, by controlling cellular processes common to a wide range of stresses, such as scavenging of oxidative burst and maintenance of turgor pressure (Choi et al. 2013; Chowdhury et al. 2017; Das et al. 2011; Weber et al. 2014; Zhu et al. 1995). However, our in vivo challenge results demonstrated extensive presence of *B. cinerea* structures in the leaf tissue of OLP lines, and only a slight effect on disease progression, restricted to the initial stages of pathogenesis. These effects may be due to insufficient expression levels, since a dosage-dependent response has been observed in in vitro (Chowdhury et al. 2015) and in vivo (Chowdhury et al. 2017) studies against fungi. Alternatively, the selective mechanism of necrotrophic pathogens, such as *B. cinerea*, against plant OLPs and chitinases (Marcato et al. 2017) may have contributed to the low manifestation of resistance in the lines expressing proteins with antimicrobial activity.

Expression of hpRNA inducing viral sequences reduces GLRaV-3 titer in grafted plants

In plants, biotechnological applications aiming antiviral protection frequently employs RNA interference (RNAi)-based mechanisms. These systems employ the genetically engineered expression of double-stranded (dsRNA) cognate to a target viral sequence to trigger endogenous antiviral pathways in the host plant (Pooggin 2017). Constructions of intron-spliced hairpin RNA with inverted repeats of a viral sequence are considered highly effective in silencing the target sequences (Smith et al. 2000). However, the engineered degradation of viral sequences was demonstrated to be unstable, due to transcriptional and post-transcriptional RNA-mediated mechanisms (Lewsey et al. 2016) and antiviral protection is not always achieved, despite the accumulation of transgene coded sRNAs (López et al. 2010; Ribeiro et al. 2007). In grapevine, genetic engineering employing sequences

of viral origin frequently results in low or unstable mRNA and protein accumulation (Gambino et al. 2005; Mauro et al. 1995; Vigne et al. 2004).

In our study, lower viral titers were observed in genetically engineered grapevine lines carrying hairpin-inducing constructs for the 3′ or 5′ termini of the coat protein gene of a local GLRaV-3 isolate, in comparison to the wild type and empty vector controls, although leaf roll symptoms and virus replication were detected in hpRNA lines. The assessment of the contribution of transgene-mediated responses to viral resistance in genetically engineered grapevines is not straightforward, due to the activation of the endogenous post-transcriptional gene silencing mechanism upon virus infection (Gambino et al. 2010; Hewezi et al. 2017; Singh et al. 2012). Moreover, leafroll disease is currently associated to a complex of six viruses, designated as GLRaV-1, -2, -3, -4 (and its strains), -7 and -13, which are genetically diverse in genome size, organization, and gene content (Burger et al. 2017). The GLRaV-3 group itself is highly diverse, consisting of, at least, eight distinct clades, with significant variation in viral genome sequence, and in length of the 5′ untranslated regions (Burger et al. 2017). Genetic variants of GLRaVs are frequently found in a single plant due to the clonal propagation of grapevine, the absence of natural resistance sources in *Vitis* spp., and the intrinsically error-prone nature of viral genome replication (Burger et al. 2017). Thus, the occurrence of unaccounted for or uncharacterized viruses may have contributed to the observed leafroll symptoms. Moreover, the use of molecular methods based on partial sequence information, such as in gene-specific amplifications and serological analyses, is also considered imprecise to characterize GLRaV coinfections and mixed infections with other viruses (Burger et al. 2017). The comparison of virus titers between genetically engineered and control plants suggests that, besides the common endogenous RNA silencing mechanism triggered by grafting, the insertion of the hp-inducing construct promoted further protection but was ineffective to fully prevent replication and pathogenesis by GLRaV-3 and related variants. The actual contribution of the inserted constructs remains to be determined.

Global genome methylation correlates positively with the contents of virus-derived sequences

DNA methylation consists of an important epigenetic mechanism associated to transcriptional regulation and silencing of repetitive and mobile transposable elements (Seymour and Becker 2017). Three distinct contexts of methylation occur in plants; at the CG, CHG (where H is A, T, or C), and CHH, each requiring specific subsets of effector enzymes and cofactors (Seymour and Becker 2017). The genomic stress associated to developmental and metabolic reprograming in tissue culture promotes epigenetic changes, likely to be different depending on the genomic context (Miyao et al. 2012). The hybrid cultivar 'BRS Clara' and the *vinifera* 'Chardonnay' exhibited similar levels of global genome methylation, although complex plant genomes tend to display higher contents of methylated DNA (Seymour and Becker 2017). It is likely to be due to the small percentage of wild *Vitis* in 'BRS Clara' genome (less than 15%) and to global nature of the analysis that does not target methylation-prone sequences. In grapevine, tissue culture variants of 'Chardonnay' and 'Syrah' were demonstrated to have altered patterns of genome wide methylation, investigated by Methylation Sensitive Amplified Polymorphism (MSAP) (Baránek et al. 2010; Schellenbaum et al. 2008). In contrast, molecular markers based exclusively on DNA sequence, such as Simple Sequence Repeat (SSR) and Amplified Fragment Length Polymorphism (AFLP), were less effective to detect genomic differences between variants and the original cultivar, suggesting that tissue culture induced epigenetic changes are transient in grapevine (Schellenbaum et al. 2008). Most of the non-transient epigenetic changes induced by tissue culture techniques in *Vitis* consist of mitotically-inherited epimutations and include regions subject to methylation modifications in response to stress in other plant genomes, such as retrotransposon and methyltransferase sequences (Baránek et al. 2015). In agreement, our results demonstrated that genetic engineering was responsible for higher levels of global genome methylation in comparison to tissue culture alone. Moreover, methylation levels were positively correlated to larger portions of repetitive (transgene copy numbers) and viral sequences (promoter and gene of interest). In a similar study, genetically engineered grapevine lines, carrying the

full-length sense or antisense sequence of the CP gene of the grapevine fanleaf virus (GFLV), exhibited a weak correlation between T-DNA copy number and the accumulation of its mRNA and coat protein (Gambino et al. 2005). In a subsequent study, the authors demonstrated, employing bisulphite sequencing, that methylation of sequences of viral origin was frequent in lines with complex rearrangements of the inserted T-DNA (Gambino et al. 2010). The genetic changes caused by the insertion of exogenous sequences in plant genomes are clearly associated to epigenetic modifications, although the correlation between DNA methylation and gene expression is low, but significant (Seymour and Becker 2017).

Disease resistance expression correlates negatively to global genomic methylation

The ectopic proteins used to produce genetically modified grapevines, namely a fungal chitinase and a *Solanum nigrum* thaumatin-like protein, are part of the plant basal defense mechanism, shared by pathways triggered against necrotrophic, biotrophic and hemi-biotrophic fungi. Genetically engineered grapevines carrying similar proteins with antimicrobial activity showed variable levels of tolerance to fungal pathogens, although rarely of complete immunity (Gray et al. 2014). Accordingly, in our study the expression of CHIT or OLP delayed pathogenesis in grapevine leaves challenged with *B. cinerea*. However, symptoms and pathogen growth were observed in in vitro assays for all modified lines. Chitinase engineered grapevines were also shown to exhibit susceptibility levels like those of wild type plants (Bornhoff et al. 2005; Rubio et al. 2015). Cisgenic grapevines expressing the coding sequences of thaumatin-like proteins from 'Chardonnay' and *Vitis amurensis* exhibited delayed pathogenesis against fungal pathogens (Dhekney et al. 2011; He et al. 2017). In general, these studies demonstrated variable levels of resistance among lines and biological challenge conditions, such as in vitro, greenhouse or field assays (Gray et al. 2014; He et al. 2017; Rubio et al. 2015). Genetic engineering aiming to induce silencing of virus-derived sequences was extensively used in grapevine (Gray et al. 2014; Maliogka et al. 2015), although mRNA and protein accumulation was demonstrated to be low or unstable, and to show a weak correlation with disease expression (Gambino et al. 2005; Mauro

et al. 1995; Vigne et al. 2004). The inconsistent patterns of resistance manifestation in genetically engineered grapevines suggest that uncharacterized factors also contribute to the response phenotype and epigenetic modifications are currently considered contributors to the phenotype of genetically modified plants (Ji et al. 2015; Lewsey et al. 2016; Matzke et al. 2015).

In *Arabidopsis thaliana* and rice, the loss of function of genes crucial to genome methylation pathways only affects marginally the global patterns of gene expression (review in Seymour and Becker 2017), suggesting that DNA methylation is responsible for the control of the expression of a small number of endogenous loci. However, genomic feature and position are also known to influence the associations between DNA methylation and gene expression (Seymour and Becker 2017). In agreement, our results of the multivariate response regression with discriminant analysis of partial least squares suggested a significant association between the global levels of genome methylation and the expression of resistance in genetically engineered plants. Moreover, our results also demonstrate that the origin of the inserted sequences is an important factor contributing to the degree of global epigenetic changes in the host genome.

The expression of the reporter gene *gusA* in genetically engineered grapevine lines was negatively correlated to the transgene copy number, the portion of introduced sequences of viral origin, and the levels of global genome methylation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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