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The interplay between hormone signaling and defense gene expression in grapevine genotypes carrying genetic resistance against *Plasmopara viticola*

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Supplementary material

Supplementary Methodology - Hormonal analysis methodology

The samples were ground using pestle and mortar in liquid nitrogen. Afterwards, 500 mg of the ground sample were transferred to a 15 mL tube containing an extraction solution (acetonitrile:methanol:formic acid 50:45:5 v/v). Then MgSO4, NaCl, Na3C6H5O7. 2H2O, C6H6Na2O7.1,5 H2O (4:1:1:0.5) and 4 mL of extraction solution were added to each tube, maintained in a pendulum shaker (Labnet Rocker 25) for 24 h at 4 °C (inside a refrigerator). The samples were vortexed, and immediately centrifuged at 3026 g for 30 min at 4 °C. The supernatant was transferred to another 15 mL tube and kept at -20 °C. Three mL of the extraction solution was added to each tube with the precipitate from the first extraction. The samples were vortexed and returned to the pendulum shaker for 24 h twice, then the samples were centrifuged at 3026 g for 30 min at 4 °C. The supernatant was concentrated under vacuum, using Concentrator Plus (Eppendorf, USA). The pellets were suspended in 1 mL of 1 mol·L^{-1} formic acid and vortexed. The purification of resuspended extracts was carried out using SPE (solid-phase extraction) columns (Waters Oasis MCX 150 mg, USA), according to the manufacturer's instructions. The eluent of each sample was newly concentrated, the pellet was resuspended in 200 µL methanol and filtered through a 0.22 µm PTFE filter. In each set of processed samples, a vial with a standard solution of 1000 ng·mL^{-1} of the set of hormones analyzed, and a vial with only Milli-Q water, were used as references. Samples were then analyzed by LC–MS/MS, consisting of an Acquity UPLC™ System (Waters, USA) quaternary pump equipped with an autosampler (7.5 µL of injection volume). The column used was Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 µm) (Waters, USA) and the mobile phase in the chromatographic separation consisted of eluent A (1 mM ammonium acetate and 0.1 % formic acid in water) and eluent B (1 mM ammonium acetate and 0.1 % formic acid in acetonitrile). The gradient used was 1 % B until 1 min, followed by a linear increase up to 6 min reaching 38 % B, followed by 100 % B until 8.5 min as a cleaning step, and finally changing to the initial 1 % B condition up to 9 min. The flow rate was 0.3 mL·min^{-1} and the column temperature was 40 °C. A Waters Xevo™ triple quadrupole mass spectrometer system (MS/MS) with an ESI interface was used in tandem MS analyses with the following conditions: capillary voltage, 2.7 kV; source temperature, 150 °C; desolvation temperature, 400 °C; desolvation gas flow, 800 L·h^{-1}; cone gas flow, 50 L·h^{-1}. Cone voltage (V, in +/- modes) and collision energy (eV) were optimized to MS/MS detection of each hormone (fragmentation patterns), as follows: IAA (176>130 m/z), +18 V, 12 eV; IAA-d2 (178>132 m/z), +18 V, 12 eV; Z (220>136 m/z), +18 V, 12 eV; tZR (352>220 m/z), +25 V, 22 eV; EBL (481,5>445,1 m/z), +20 V, 10 eV; SA (137>93 m/z), -25 V, 13 eV; JA (209>59 m/z), -24 V, 13 eV; ABA (137>93 m/z), -20 V, 12 eV; GA4 (331>257 m/z), -30 V, 20 eV; GA3 (345>239 m/z), -60 V, 14 eV. The multiple reaction monitoring (MRM) mode was applied in this analysis. Concentrations of 5, 10, 50, 100, 250, 500, and 1000 ng·mL^{-1} were prepared in three separate dilutions in methanol to obtain the standard curve, and the analysis/quantification of each dilution was performed in duplicate. The quantification was achieved using TargetLynx™ software (Waters, USA), with a limit of detection (LOD) greater than 3, and a limit of quantification (LOQ) greater than 10. The recovery efficiency and matrix effect were determined with standard spikes (1000 ng·sample^{-1} of all hormones) in a group of samples during the extraction and detection steps. All variations in recovery and matrix effect were considered in the final concentration of each hormone.

Table S1

Oligonucleide sequences for gene expression analysis, by Sybr RT-qPCR from genes related to defense to pathogens and signaling pathways

Gene	Gene function	GenBank access	Oligonucleotides forward and reverse	Reference
		Housekeeping genes		
Actinia	Housekeeping gene	AC969944	F: CTTGCATCCCTCAGCACCTT R: TCCTGTGGACAATGGATGGA	Reid *et al*. 2006
EF1-α	Housekeeping gene	XM_002279562.2	F: CTCCAAGTCCAGGTATGATG R: CAGAGATTGGAACAAAGGGG	Wang *et al.* 2018
GAPDH	Housekeeping gene	EF192466	F: TCAAGGTCAAGGACTCTAACACC R: CCAACAACGAACATAGGAGCA	Monteiro *et al.* 2013
Ubiquitin	Housekeeping gene	EC929411	F: GAGGGTCGTCAGGATTTGGA R: GCCCTGCACTTACCATCTTTAAG	Reid *et al*. 2006
		Jasmonic acid related genes		
JAZ 1	Repressor of JA responses	XM_002272327.3	F: CAACCCAAAGCTCAACAAAG R: TAAGTGGGAGTGGACAAGAT	Guerreiro *et al.* 2016
JAZ 3	Repressor of JA responses	XM_002282652.2	F: TCCCTCCTGTAAGTCCCAAT R: TCCCCATAAAACCATCACCT	Guerreiro *et al.* 2016
MYC 2	Transcriptional activator of light, ABA, and JA signaling pathways	XM_002280217.2	F: ATGCATTGCGAGCTGTTGTG R: TCTGCCTCGGTGTTAGTTTC	Guerreiro *et al.* 2016
NINJA	Negative regulator of JA responses	XM_002283943.2	F: AAATTCGGGGGATCTGGTTC R: TGGATTGGCATGCTCTTCAC	Guerreiro *et al.* 2016
TOPLESS	Negative regulator of jasmonate responses	XM_002268229.1	F: TCGGGATGGATGATTCTACA R: GGCAAGGCCAGTTATTCTC	Guerreiro *et al.*. 2016
PR10	Pathogenesis-related protein	HS075818	F: GTTTTGACTGACGGCGTTGA R: TGGTGTGGTACTTGCTGGTGTT	Guerreiro *et al.* 2016
		Salicylic acid related genes		
NPR 1	Positive regulate of SA signaling	XM_002281439.2	F: ATGGATGCCGATGACTTA R: TCCTTGTACCTCCTCTTCTT	Guerreiro *et al.* 2016
PR 1	Defense against pathogens	XM_002273752.2	F: AAAAATGGGGTTGTGTAGGAG R: TGTTGTGAGCATTGAGGTAGT	Guerreiro *et al.* 2016
WRKY70	Regulator of SA and JA pathway	XM_002275365.2	F: GCCACCATACTTGCAGAGAT R: CAGACCCAACCATATTATTAG	Guerreiro *et al.* 2016
AtMYB44	Regulator of SA and JA pathway	XM_002284979.2	F: CAACGGTTTCGGGTCATAAT R: GTTCTCGGCACTGGTCTAT	Guerreiro *et al.* 2016
		Stilbenes related genes		
STS	Phytoalexins production	DQ459351.1	F: GAGTTCTTGTGGTGTGCTCTG R: GCTGCTGAGACAAGCTGGAAG	Wang *et al.* 2018
ROMT	Biosynthesis of pterostilbene	FM178870.1	F: CTCGACCCAATTTTAACTAAACCA R: TCATTGAAGGAATTGTTGAGCTG	Wang *et al.* 2018
GT	O-glucosyltransferase	DQ832169.1	F: GAAGTATGATGAAATCGCCAGC R: TATTCAATGACTTCGGGTTCCAG	Wang *et al.* 2018

Table S2

The kinetic of plant hormones expression in 0, 3, 5, and 7 days post inoculation (DPI) with *Plasmopara viticola* oospore, in in vitro cultivated leaves of *V. vinifera* carrying different genetic resistance loci, *Rpv3-1+Rpv3-2*, *Rpv3-3+Rpv10*, *Rpv3-1*, and without genetic resistance genes (susceptible). Means followed by the same capital letters in the columns and lowercase letters in the lines, do not present statistically differences by Tukey test (α = 0.05)

	0 DPI	3 DPI	5 DPI	7 DPI
		Indole-3-acetic acid (ng g^{-1})		
Chardonnay (*suceptible*)	2.68 aAB	1.41 aAB	3.74 aAB	2.01 aAB
Regent (*Rpv3-1*)	1.70 aB	0.94 aB	0.84 aB	0.00 aB
GF15 (*Rpv1+Rpv3-1*)	1.67 aA	3.79 aA	3.24 aA	6.46 aA
Calardis Blanc (*Rpv3-1+Rpv3-2*)	4.11 aA	3.70 aA	3.78 aA	3.53 aA
Bronner (*Rpv3-3+Rpv10*)	3.42 aAB	2.37 aAB	1.70 aAB	2.84 aAB
		Abscisic acid (ng g^{-1})		
Chardonnay (*suceptible*)	773.12 aA	411.79 bA	321.17 bA	371.95 bA
Regent (*Rpv3-1*)	491.60 aB	360.74 aA	281.93 aA	490.26 aA
GF15 (*Rpv1+Rpv3-1*)	53.50 aC	160.82 aA	125.02 aA	290.66 aA
Calardis Blanc (*Rpv3-1+Rpv3-2*)	306.42 aB	330.38 aA	223.12 aA	297.14 aA
Bronner (*Rpv3-3+Rpv10*)	520.19 aB	296.35 abA	211.51 bA	446.75 aA
		trans-Zeatin-Ribose (ng g^{-1})		
Chardonnay (*suceptible*)	2.62 aA	2.09 aA	1.73 aA	2.21 aA
Regent (*Rpv3-1*)	2.03 aA	2.20 aA	2.01 aA	1.53 aA
GF15 (*Rpv1+Rpv3-1*)	2.12 aA	2.20 aA	1.71 aA	2.32 aA
Calardis Blanc (*Rpv3-1+Rpv3-2*)	0.80 aA	1.29 aA	1.91 aA	0.62 aA
Bronner (*Rpv3-3+Rpv10*)	1.84 aA	1.95 aA	1.91 aA	1.51 aA
		Salicylic acid (ng g^{-1})		
Chardonnay (*suceptible*)	412.44 aBC	340.68 aB	434.58 aB	401.31 aB
Regent (*Rpv3-1*)	621.66 aB	503.43 aB	302.49 aB	486.81 aB
GF15 (*Rpv1+Rpv3-1*)	223.21 aC	261.75 aB	247.68 aB	324.43 aB
Calardis Blanc (*Rpv3-1+Rpv3-2*)	2378.28 aA	1698.81 bA	1144.06 cA	1023.19 cA
Bronner (*Rpv3-3+Rpv10*)	467.59 aBC	310.50 aB	200.97 aB	431.76 aB
		Jasmonic acid (ng g^{-1})		
Chardonnay (*suceptible*)	31.83 aA	3.31 bB	23.16 aAB	21.12 aAB
Regent (*Rpv3-1*)	6.23 aB	0.92 aB	5.88 aB	4.86 aB
GF15 (*Rpv1+Rpv3-1*)	5.56 bB	30.79 aA	35.05 aA	28.72 aA
Calardis Blanc (*Rpv3-1+Rpv3-2*)	7.90 aB	14.10 aAB	8.44 aB	8.43 aB
Bronner (*Rpv3-3+Rpv10*)	12.25 aB	1.49 aB	18.08 aAB	8.55 aB
		Zeatin (ng g^{-1})		
Chardonnay (*suceptible*)	0.79 aA	1.37 aA	2.71 aA	2.21 aA
Regent (*Rpv3-1*)	1.21 aA	1.27 aA	1.43 aA	1.59 aA
GF15 (*Rpv1+Rpv3-1*)	0.47 aA	0.55 aA	1.48 aA	2.13 aA
Calardis Blanc (*Rpv3-1+Rpv3-2*)	1.32 aA	1.12 aA	1.29 aA	2.01 aA
Bronner (*Rpv3-3+Rpv10*)	1.31 aA	0.81 aA	1.42 aA	1.54 aA

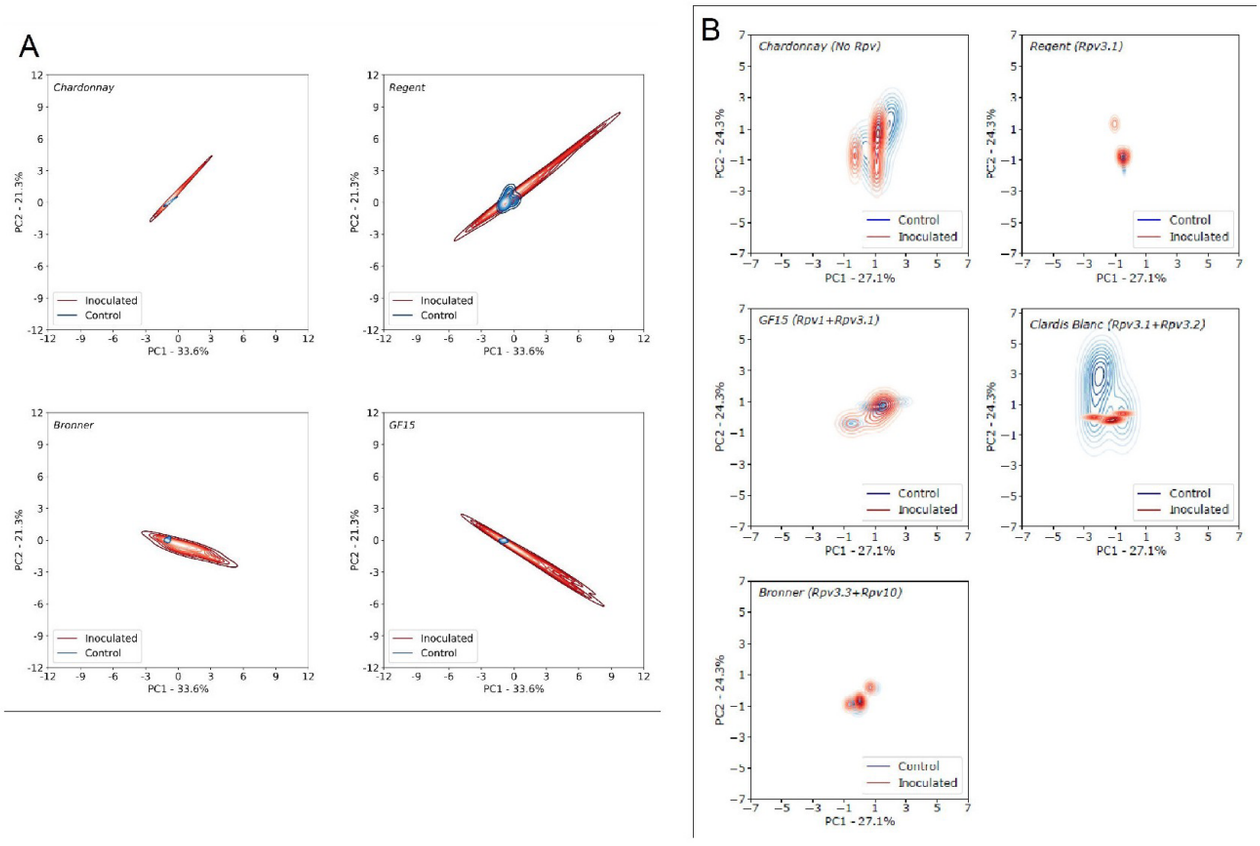

Fig. S1: (**A**) Kernel density estimation according to the two principal components (PC1 and PC2) derived from PCA, showing gene expression levels in grapevines with no treated leaves (control – blue) and leaves inoculated with *Plasmopara viticola* (inoculated – red) in genotypes with different resistance levels: 'Bronner (*Rpv3-3+Rpv10*), GF15 (*Rpv1+Rpv3-1*), 'Regent' (*Rpv3-1*) and 'Chardonnay' (susceptible). (**B**) Kernel density estimation according the two principal components (PC1 and PC2) derived from PCA, showing the hormonal concentration for salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), gibberellic acid 4 (GA4) and zeatin (Z) in grapevines with no treated leaves (control – blue) and inoculated leaves, with *Plasmopara viticola* (inoculated – red) in genotypes with different resistance levels: 'Calardis blanc' (*Rpv3-1+Rpv3-2*), 'Bronner' (*Rpv3-3+Rpv10*), GF15 (*Rpv1+Rpv3-1*), 'Regent' (*Rpv3-1*) and 'Chardonnay' (susceptible).

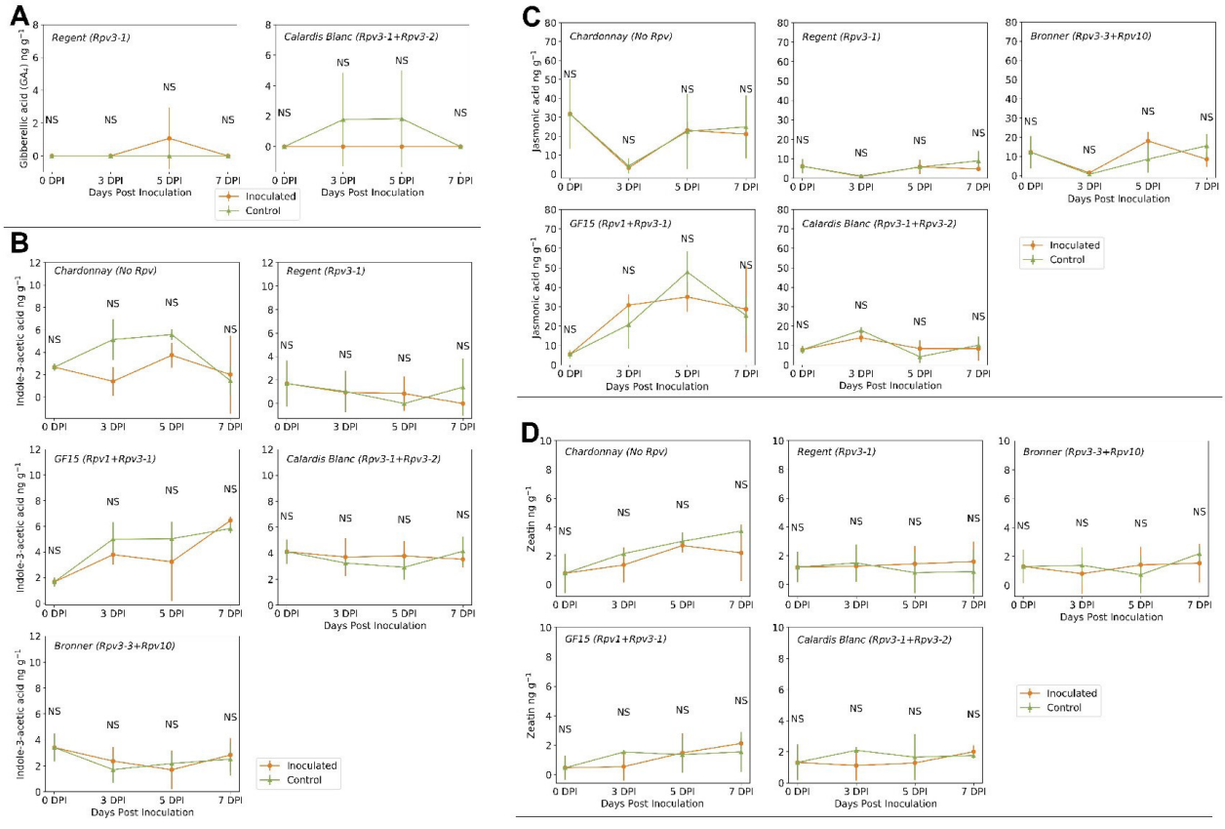

Fig. S2: (**A**) Kinetics of gibberellic acid 4 (GA4) concentration in grape leaf tissue inoculated with *Plasmopara viticola* in genotypes with different levels of resistance against downy mildew. Pairwise comparison controlled and inoculated conditions, columns with identical letters in the same day are not statistically different by t test ($P < 0.05$). (**B**) Kinetics of indoleacetic acid (IAA) concentration in grape leaf tissue inoculated with *P. viticola* in genotypes with different levels of resistance against downy mildew and susceptible ('Chardonnay' – no *Rpv*). Pairwise comparison controlled and inoculated conditions, columns with identical letters in the same day are not statistically different by t test ($P < 0.05$). (**C**) Kinetics of jasmonic acid (JA) concentration in grape leaf tissue inoculated with *P. viticola* in genotypes with different levels of resistance against downy mildew and susceptible ('Chardonnay' – no *Rpv*). Pairwise comparison controlled and inoculated conditions, columns with identical letters in the same day are not statistically different by t test ($P < 0.05$). (**D**) Kinetics of zeatin (Z) concentration in grape leaf tissue inoculated with *P. viticola* in genotypes with different levels of resistance against downy mildew and susceptible ('Chardonnay' – no *Rpv*). Pairwise comparison controlled and inoculated conditions, columns with identical letters in the same day are not statistically different by t test ($P < 0.05$).