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Laser Microdissection of Grapevine Leaves Reveals Site-Specific Regulation of Transcriptional Response to *Plasmopara viticola*

Luisa Lenzi[1,2], Carla Caruso[2], Pier Luigi Bianchedi[3], Ilaria Pertot[1] and Michele Perazzolli[1,*]

[1]Research and Innovation Center, Fondazione Edmund Mach (FEM), Via E. Mach, 1, 38010 S. Michele all'Adige, Italy
[2]Department of Ecological and Biological Sciences, University of Tuscia, Via San Camillo de Lellis, 01100 Viterbo, Italy
[3]Technology Transfer Center, Fondazione Edmund Mach (FEM), Via E. Mach, 1, 38010 S. Michele all'Adige, Italy
*Corresponding author: E-mail, michele.perazzolli@fmach.it; Fax, 00390461615765.
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Grapevine is one of the most important fruit crops in the world, and it is highly susceptible to downy mildew caused by the biotrophic oomycete *Plasmopara viticola*. Gene expression profiling has been used extensively to investigate the regulation processes of grapevine–*P. viticola* interaction, but all studies to date have involved the use of whole leaves. However, only a small fraction of host cells is in contact with the pathogen, so highly localized transcriptional changes of infected cells may be masked by the large portion of non-infected cells when analyzing the whole leaf. In order to understand the transcriptional regulation of the plant reaction at the sites of pathogen infection, we optimized a laser microdissection protocol and analyzed the transcriptional changes in stomata cells and surrounding areas of grapevine leaves at early stages of *P. viticola* infection. The results indicate that the expression levels of seven *P. viticola*-responsive genes were greater in microdissected cells than in whole leaves, highlighting the site-specific transcriptional regulation of the host response. The gene modulation was restricted to the stomata cells and to the surrounding areas of infected tissues, indicating that the host response is mainly located at the infection sites and that short-distance signals are implicated. In addition, due to the high sensitivity of the laser microdissection technique, significant modulations of three genes that were completely masked in the whole tissue analysis were detected. The protocol validated in this study could greatly increase the sensitivity of further transcriptomic studies of the grapevine–*P. viticola* interaction.

Keywords: Gene expression • Laser microdissection • Oomycetes • Plant–pathogen interactions • *Plasmopara viticola* • Stomata • *Vitis vinifera*.

Abbreviations: CellS, cellulose synthase; CHI, chalcone isomerase; d, days; GLP, germin-like protein; hpi, hours post-inoculation; LMD, laser microdissection; LSU, large ribosomal subunit; OSM, osmotin; PR, pathogenesis-related; r.p.m., revolution per minute; SE, standard error; TLP, thaumatin-like protein.

Introduction

Downy mildew caused by the biotrophic oomycete *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni is one of the most economically significant grapevine diseases worldwide, particularly in warm, wet climates (Gessler et al. 2011). *Plasmopara viticola* attacks all green parts of the grapevine where functional stomata are present (in particular, leaves and clusters), and it penetrates only these natural openings (Gessler et al. 2011). When leaves are wet, zoospores are released from sporangia into the water film and swim towards the stomata where they encyst (Unger et al. 2007, Díez-Navajas et al. 2008). Subsequently, encysted zoospores form a germ tube that penetrates into the substomatal cavity and develops the primary hypha that branches to form a complex mycelial network (Unger et al. 2007, Díez-Navajas et al. 2008). Then, *P. viticola* hyphae expand into the intercellular spaces of the mesophyll tissue and differentiate specialized structures known as haustoria (Unger et al. 2007, Liu et al. 2015). Haustoria development involves localized degradation of the plant cell wall and invagination of the plasma membrane, creating intimate contact between the pathogen and the host (Jones and Dangl 2006). These highly specialized structures of biotrophic oomycetes and fungi play an essential role in nutrient acquisition from the plant cells and allow intense exchanges of signals that redirect the host metabolism and suppress the defense reaction (Voegele and Mendgen 2003, Dodds et al. 2009). In particular, pathogenicity effector proteins are released by the pathogen to manipulate host cells and establish a parasitic relationship (Voegele and Mendgen 2003, Kamoun 2006, Dodds et al. 2009). The molecular basis of grapevine and *P. viticola* interaction is still poorly understood, and only three effector genes have been identified in *P. viticola* (Mestre et al. 2012). However, it is assumed by comparison with similar pathosystems that the establishment of compatibility is achieved through the secretion of effector molecules, which can act as virulence factors in suppressing host defenses (Milli et al. 2012) and which can manipulate host cell metabolism to maximize energy recruitment (Gamm et al. 2011).

The wine industry relies predominantly on *Vitis vinifera*, which is highly susceptible to downy mildew (Gessler et al. 2011). Resistance traits are present in some other species of the genus *Vitis*, which are commonly used in plant breeding for resistance to *P. viticola* (Kortekamp 2006, Polesani et al. 2010, Wu et al. 2010, Malacarne et al. 2011, Figueiredo et al. 2012, He et al. 2013). A better understanding of the mechanisms responsible for compatible and incompatible interaction

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between *Vitis* spp. and *P. viticola* will help breeding programs to develop resistant varieties. Restriction of pathogen growth in resistant grapevines is mainly a post-infection phenomenon (Polesani et al. 2010) that begins when the first haustoria establish intimate contact with plant cells (Unger et al. 2007, Díez-Navajas et al. 2008, Polesani et al. 2010, Liu et al. 2015). Transcriptional responses to *P. viticola* also occur in susceptible grapevines, and they have been associated with a weak and abortive defense response at early infection stages (Polesani et al. 2010), followed by the establishment of a compatible interaction (Polesani et al. 2008, Legay et al. 2010, Gamm et al. 2011, Perazzolli et al. 2012). Grapevine response to downy mildew is characterized by the induction of genes involved in transcription regulation, such as a NAC transcription factor (Polesani et al. 2010), and defense response, including synthesis of pathogenesis-related (PR) proteins and antimicrobial compounds (Kortekamp 2006, Polesani et al. 2010, Wu et al. 2010, Malacarne et al. 2011, Figueiredo et al. 2012, He et al. 2013). PR proteins are associated with plant resistance to pathogens (van Loon et al. 2006). In particular, grapevine *PR-2* and *PR-4* genes are induced in response to *P. viticola* inoculation and code for β-glucanase and chitinase, respectively (Kortekamp 2006, Perazzolli et al. 2011). Likewise, osmotins (OSMs) and thaumatin-like proteins (TLPs) belong to the *PR-5* family (van Loon et al. 2006), and exhibit strong inhibitory effects on germination and growth of the major pathogenic fungi of grapevine (Monteiro et al. 2003). As members of the PR-16 class (Park et al. 2004), seven genes of the germin-like protein (GLP) family were identified in grapevine and two of them, *GLP-2* and *GLP-7*, were induced by *P. viticola* inoculation (Godfrey et al. 2007). Phytoalexins and phenolic derivatives are essential antimicrobial compounds produced following downy mildew infection, and are considered as markers of grapevine resistance (Slaughter et al. 2008, Malacarne et al. 2011). These defense molecules are synthesized by the phenylpropanoyd pathway, which involves several enzymes, including a *P. viticola*-induced chalcone isomerase (CHI) (Legay et al. 2010). The grapevine response to downy mildew also includes the induction of a cellulose synthase (*CellS*) gene, which may be involved in the supply of precursors for pathogen metabolism (Polesani et al. 2008, Polesani et al. 2010).

Several studies have characterized the transcriptional changes of grapevine leaves occurring in response to *P. viticola* inoculation by analyzing RNA extracted from whole leaves (Kortekamp 2006, Polesani et al. 2008, Legay et al. 2010, Polesani et al. 2010, Wu et al. 2010, Gamm et al. 2011, Perazzolli et al. 2012, Li et al. 2015, Merz et al. 2015). However, only a small fraction of leaf cells is in contact with the pathogen at early infection stages, and the large portion of non-infected cells could mask or dilute highly localized transcriptional changes related to the establishment of haustoriated cells. On the other hand, little is known about the local plant response to biotrophic pathogens and the potential for different transcriptional regulations of parasitized plant cells and leaf tissues far from the infection sites (Coker et al. 2015). Laser microdissection (LMD) is a powerful methodology for precisely isolating specific cell groups or single cells from heterogeneous tissues and also allows single-cell gene expression analyses (Emmert-Buck et al. 1996). The LMD approach has been successfully used to study the transcriptional reprogramming of host cells during plant–microbe interactions, such as with nitrogen-fixing bacteria (Damiani et al. 2012, Roux et al. 2014), pathogenic fungi (Tang et al. 2006, Chandran et al. 2010, Hacquard et al. 2010, Klug et al. 2015), phytoplasma (Santi et al. 2013), arbuscular mychorrhizal (Balestrini et al. 2007, Fiorilli et al. 2009, Gaude et al. 2012) and ectomychorrhizal fungi (Hacquard et al. 2013).

The aim of this work was to compare early grapevine response in whole leaves, in the sites of *P. viticola* infection, in surrounding areas and in distal parts not infected by the pathogen. A protocol for sample preparation was optimized to isolate leaf cells, and the expression level of downy mildew-responsive genes was investigated to highlight different expression profiles in sites closely associated with *P. viticola* and in whole leaves.

Results

Plasmopara viticola inoculation of grapevine leaves

Leaf samples were collected at 24 h post-inoculation (hpi) from water-treated leaves (uninoculated sample), from inoculated areas (local-inoculated sample) and from uninoculated areas (distal-inoculated sample) of leaves inoculated with *P. viticola* only on the left side of the primary vein (left-side inoculated leaves). In local-inoculated samples, encysted zoospores and substomatal vesicles with primary hyphae of *P. viticola* were observed at 24 hpi (**Fig. 1B, C**), and they were not detected in uninoculated samples (**Fig. 1A**). Of the total stomata, 18% had an encysted zoospore or a substomatal vesicle with primary hyphae (infected stomata) (**Fig. 1D**). *Plasmopara viticola* infection was confirmed by the development of downy mildew symptoms with heavy production of *P. viticola* sporangia on local-inoculated samples at 7 d post-inoculation (**Fig. 1E**; **Supplementary Fig. S1**). No signs of the pathogen were noticed on uninoculated samples or distal-inoculated samples.

Gene expression analysis in whole fresh leaves inoculated with *Plasmopara viticola*

Early response to downy mildew was analyzed by assessing the modulation of 10 grapevine genes known to be defense response markers (**Table 1**) at 24 hpi in uninoculated and local-inoculated samples of whole fresh leaves (**Fig. 2**). The expression levels of the *TLP-5* (**Fig. 2A**), *PR-2* (**Fig. 2B**) and *PR-4* (**Fig. 2C**) genes were induced 3.1-, 3.8- and 2.9-fold in local-inoculated samples, respectively. Likewise, *P. viticola* induced the expression of the *GLP-7* (6.6-fold; **Fig. 2D**), *OSM-1* (3.8-fold; **Fig. 2E**) and *TLP-4* (2.9-fold; **Fig. 2F**) genes in whole fresh leaves. The expression levels of the *GLP-2*, *NAC*, *CHI* and *CellS* genes were not affected by *P. viticola* in whole fresh leaves of in vitro grown plants (**Fig. 2G–J**).

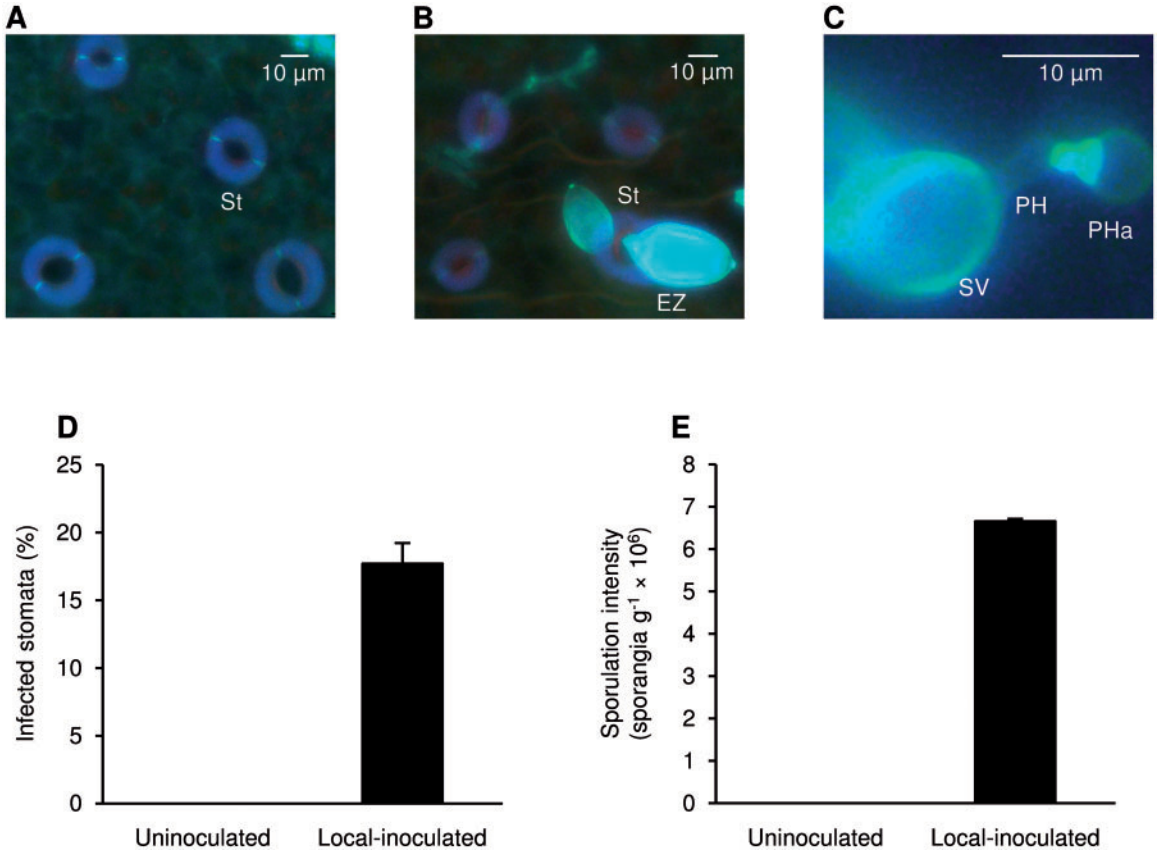

Fig. 1 Infection of *Plasmopara viticola* on grapevine leaves of in vitro grown plants. Leaves were inoculated with *P. viticola* (local-inoculated) or treated with water (uninoculated) under sterile conditions. *P. viticola* infection was assessed on (A) uninoculated and (B, C) local-inoculated samples at 24 hpi by epifluorescence microscopy after aniline blue staining. (D) Percentage of infected stomata showing encysted zoospores or substomatal vesicles with primary hyphae and primary haustoria, and (E) disease severity expressed as number of *P. viticola* sporangia per gram of leaf fresh weight were assessed at 24 hpi and at 7 d post-inoculation, respectively. Representative microscopic pictures of encysted zoospores (EZ), substomatal vesicles (SV) with primary hyphae (PH) and primary haustoria (PHa) near stomata (St) cells are shown. An *F*-test revealed non-significant differences between experiments ($P = 0.2$), and data from the two experiments were pooled. Mean scores and SEs of 10 replicates pooled from two experiments are presented.

Laser microdissection of stomata and surrounding cells from grapevine leaves

The LMD protocol was optimized by comparing two fixative solutions: ethanol–acetic acid (3 : 1) and 100% acetone. Although both fixative solutions provided adequate preservation of tissue morphology and allowed proper microdissection of stomata cells (data not shown), high-quality RNA was obtained only from microdissected cells of acetone-fixed leaves (**Supplementary Fig. S2A**). On the other hand, degraded RNA (no evident rRNA bands) was obtained from leaves fixed in the ethanol–acetic acid solution. High-quality RNA was present in acetone-fixed leaves for up to 14 d of storage at −20°C. The minimum quantity of fixed leaf tissue required to extract a sufficient amount of RNA (>4 ng) was 300 microdissected stomata cells. Acetone-fixed leaves were used in the following experiments.

Regulation of grapevine response at *P. viticola* infection sites was assessed on samples of 300 microdissected stomata cells, corresponding to a total area of 0.7–0.8 mm^2 of leaf tissue, as well as on samples of an equivalent area of microdissected surrounding cells (**Fig. 3**). LMD was carried out directly on whole fixed leaves without specimen sectioning by cryostat or microtome. Stomata cells were microdissected from uninoculated, local- and distal-inoculated samples, while surrounding cells were collected from uninoculated and local-inoculated samples. Extractions from whole fixed leaves after LMD confirmed that procedures of sample preparation and microdissection did not alter the quality of total RNA (**Supplementary Fig. S2B**). After RNA extraction, protocols for RNA amplification and cDNA synthesis were optimized for microdissected cells and whole fixed leaves, and the absence of DNA contamination was confirmed for all samples (**Supplementary Fig. S3**).

Quantification of *Plasmopara viticola* in cDNA samples

The presence of *P. viticola* was detected in the cDNA samples of whole fresh (**Fig. 4A**) and fixed (**Fig. 4B**) leaves of local-inoculated samples by real-time PCR analysis of the large ribosomal subunit (*LSU*) gene. As expected, no amplification of the *P. viticola LSU* gene was obtained from uninoculated or

Table 1 Primer sequences for quantitative real-time PCR expression analysis

Gene abbreviation	Gene name	Accession number[a]	Grapevine gene[b]	Primer sequence[c]	Reference[d]
PR-2	Pathogenesis-related protein 2	AJ277900	VIT_208s0007g06060	For: GTTATTTCAGAGAGTGGTTGGC Rev: AACATGGCAAACACGTAAGTCT	Perazzolli et al. (2011)
PR-4	Pathogenesis-related protein 4	CF074510	VIT_214s0081g00030	For: CAGGCAACGGTGAGAATAGT Rev: ACCACAGTCCACAAACTCGTA	Perazzolli et al. (2011)
OSM-1	Osmotin 1	XM_002282928.2	VIT_202s0025g04310	For: CGCTGCGCTAAAGACTACC Rev: AAAAACCTTGAGTAATCTGTAGCA	Perazzolli et al. (2012)
TLP-4	Thaumatin-like protein 4	XM_002282957.1	VIT_202s0025g04290	For: CACTGTTTTCAGGACCGATG Rev: GGGCATGTAAAGGTGCTTGT	Santi et al. (2013)
TLP-5	Thaumatin-like protein 5	XM_002283046.2	VIT_202s0025g04270	For: CTAGGGTGCTTTTGAGTCCA Rev: CGTAGAAAAGTTGTTGCATGAG	Santi et al. (2013)
GLP-7	Germin-like protein 7	NM_001281015.1	VIT_214s0128g00570	For: GGGTGTCAGTGGCTCGTATC Rev: CGGTGTTCAGGGTTGGATGT	Godfrey et al. (2007)
GLP-2	Germin-like protein 2	NM_001280985.1	VIT_214s0060g00120	For: CGAGTTGGATGTGGGGTTCA Rev: GACTTCGCCGTTGTTCTTCT	Godfrey et al. (2007)
NAC	Transcription factor *NAC*	Q52QR5	VIT_201s0026g02710	For: AACTGCCATGCCAATACAAC Rev: AACCAAGCCTAATCACTGAA	Polesani et al. (2008)
CHI	Chalcone isomerase	NM_001281104.1	VIT_213s0067g03820	For: AACTTCTGGTAGGGACCCATCT Rev: GAAGGATGAAACCTTCCCACCA	Legay et al. (2010)
CellS	Cellulose synthase-like protein D3	XM_010664727.1	VIT_202s0012g02190	For: CAGACGAAGACTTCGCTGAT Rev: GGAGTCCATGAGTTGCTGTA	Polesani et al. (2008)
Act	Actin	XM_010659103.1	VIT_212s0178g00200	For: ATTCCTCACCATCATCAGCA Rev: GACCCCCTCCTACTAAAACT	Polesani et al. (2010)
LSU	Large ribosomal subunit[e]	KM279688	-	For: GTGGTCAGTATGGGCACTTG Rev: GCGACTCAAAGAAAACGGGT	Gindro et al. (2014)

[a] Accession number in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov).
[b] Grapevine gene code of the V2 version (http://genomes.cribi.unipd.it/grape/).
[c] Sequences of the forward (For) and reverse (Rev) primers. References of primer pairs are: *PR-2* and *PR-4* (Perazzolli et al. 2011); *TLP-4* and *TLP-5* (Santi et al. 2013); *OSM-1* (Perazzolli et al. 2012); *NAC* and *CellS* (Polesani et al. 2008); *CHI* (Legay et al. 2010); *Act* (Polesani et al. 2010).
[d] References of gene markers of the grapevine defence response.
[e] The large ribosomal subunit (LSU) gene of *P. viticola* was used to detect the presence of the pathogen in leaf tissues.

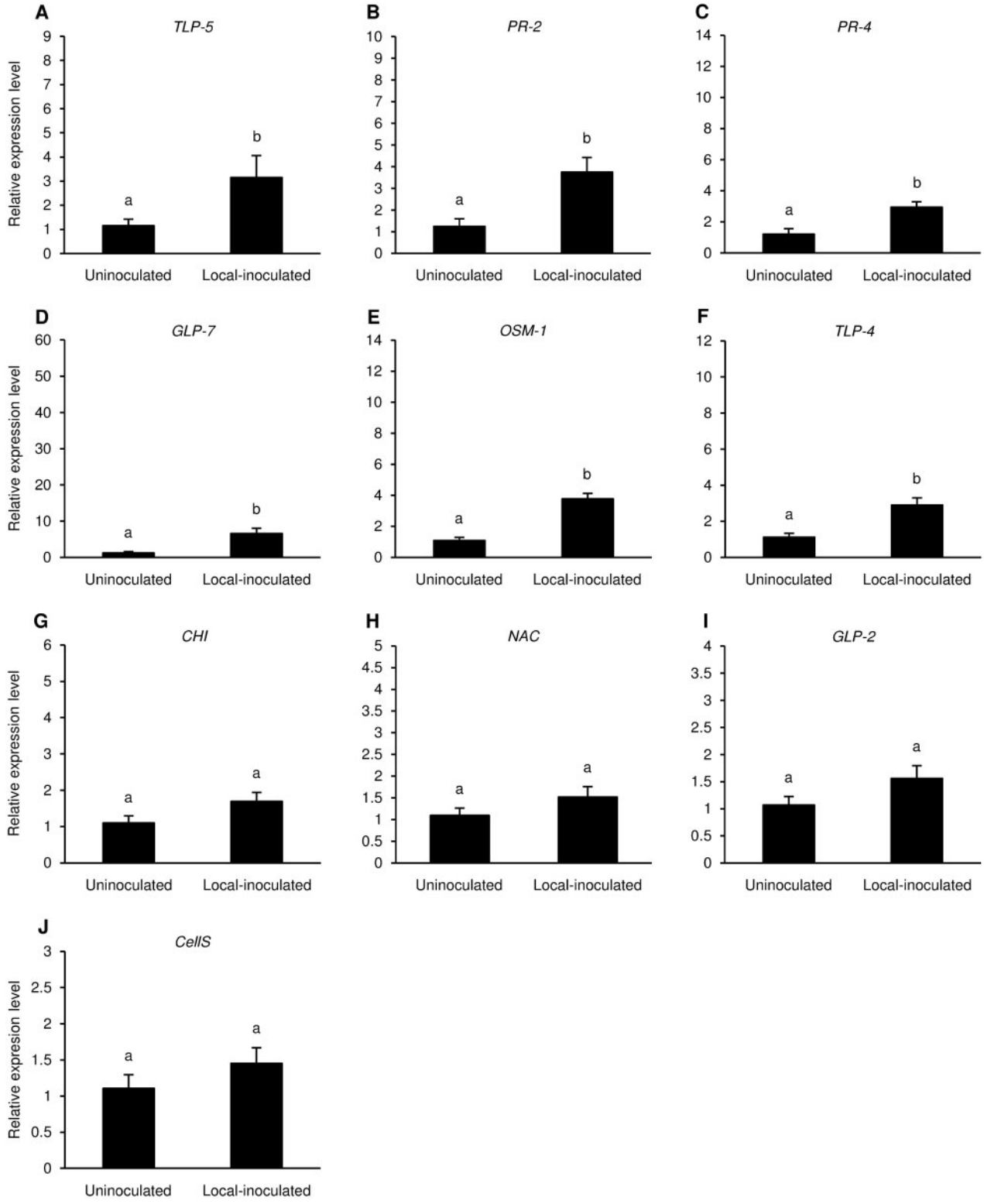

Fig. 2 Gene expression analysis of whole fresh leaves of in vitro grown grapevines. Relative expression levels of genes encoding (A) thaumatin-like protein 5 (*TLP-5*), (B) pathogenesis-related protein 2 (*PR-2*), (C) pathogenesis-related protein 4 (*PR-4*), (D) germin-like protein 7 (*GLP-7*), (E) osmotin 1 (*OSM-1*), (F) thaumatin-like protein 4 (*TLP-4*), (G) chalcone isomerase (*CHI*), (H) NAC transcription factor (*NAC*), (I) germin-like protein 2 (*GLP-2*) and (J) cellulose synthase (*CellS*) were assessed by real-time PCR analysis. Leaves were treated with water (uninoculated) or inoculated with *Plasmopara viticola* (local-inoculated) under sterile conditions, and collected at 24 hpi. Relative expression levels (fold change) were calculated using grapevine *Actin* as the constitutive gene for normalization, and data were calibrated on the uninoculated sample. An *F*-test revealed non-significant differences between experiments (*P*- values ranged from 0.2 to 0.8 for the genes tested), and data from the two experiments were pooled. Mean levels of relative expression and SEs from 7–9 replicates pooled from two experiments are presented for each sample. For each gene, different letters indicate significant differences according to Fisher's test ($\alpha = 0.05$).

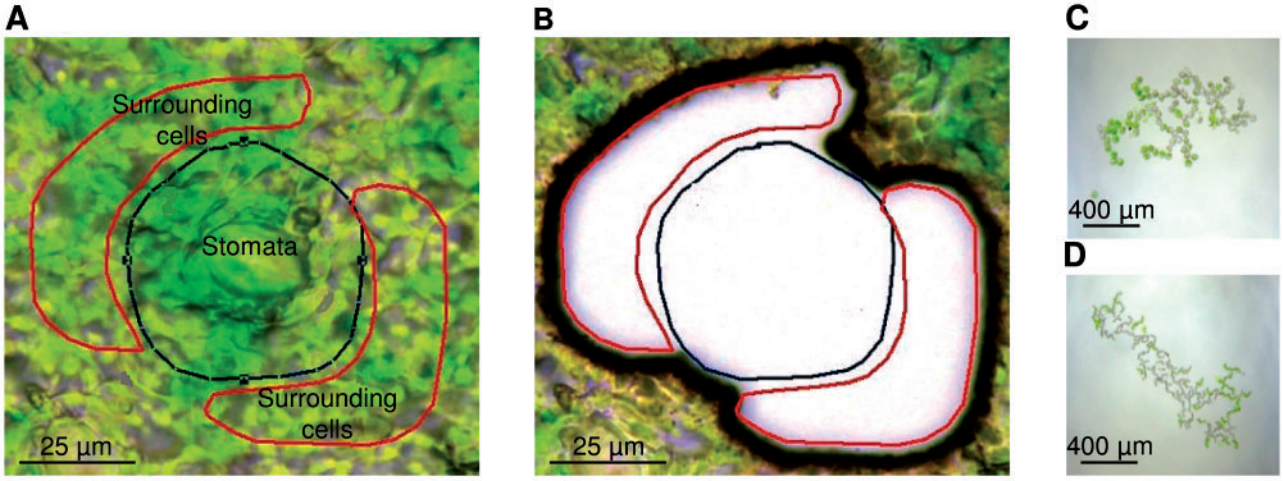

Fig. 3 Laser microdissection of stomata and surrounding cells from leaves of in vitro grown grapevines. Abaxial surface of a grapevine leaf fixed in 100% acetone with stomata (blue line) and surrounding (red line) cells selected on a Leica LMD7000 instrument (A) before and (B) after laser microdissection. Visualization of microdissected (C) stomata cells and (D) surrounding cells collected in PCR tube caps after laser microdissection.

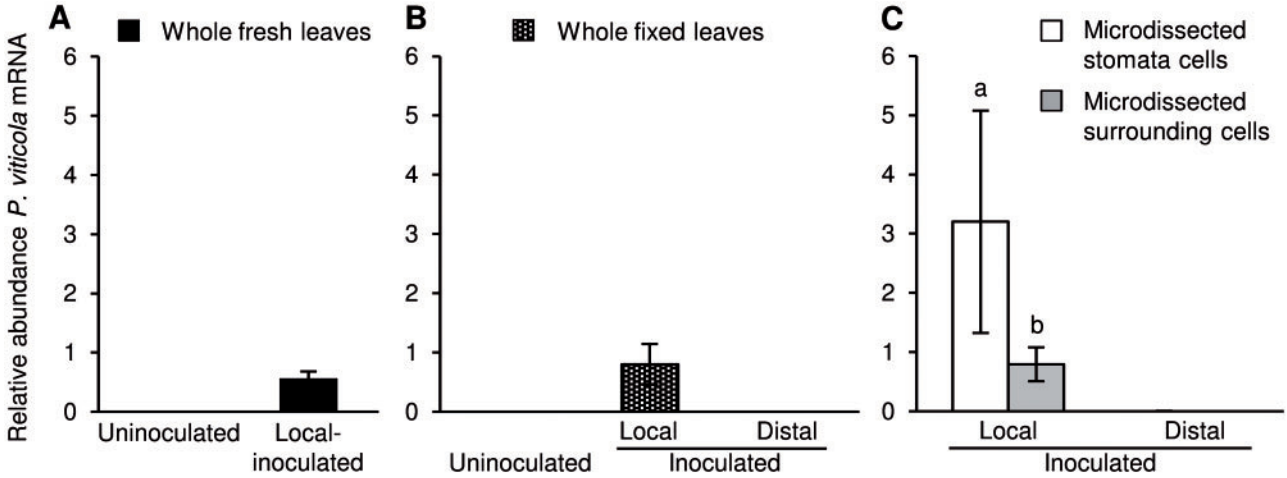

Fig. 4 Detection of *Plasmopara viticola* in grapevine leaves. *P. viticola* development was assessed in (A) whole fresh leaves, (B) whole fixed leaves and (C) microdissected stomata and surrounding cells of in vitro grown grapevines. Leaves were treated with water (uninoculated) or inoculated with *P. viticola* (inoculated) under sterile conditions, and samples were collected at 24 hpi from the inoculated (local-inoculated) or uninoculated (distal-inoculated) area of left-side inoculated leaves. *P. viticola* development was estimated by real-time PCR of the expression of the large ribosomal subunit (*LSU*) gene of *P. viticola* and normalized to the transcription level of the grapevine *Actin* gene. An *F*-test revealed non-significant differences between experiments ($P = 0.3$), and data from the two experiments were pooled. Mean levels and SEs of relative *P. viticola* quantification from 6–9 replicates pooled from two experiments are presented for each sample.

distal-inoculated samples of whole fresh or fixed leaves. Likewise, the presence of *P. viticola* was not detected in microdissected stomata or surrounding cells of distal-inoculated samples (**Fig. 4C**). The *P. viticola LSU* gene was amplified from local-inoculated samples, and its signal was 4-fold greater in microdissected stomata than in microdissected surrounding cells.

Gene expression analysis in microdissected stomata and surrounding cells of leaves inoculated with *Plasmopara viticola*

The molecular response of grapevine to *P. viticola* at the infection sites was assessed with gene expression analysis of the selected marker genes (**Table 1**) in microdissected stomata and surrounding cells of uninoculated, local- and distal-inoculated samples (**Fig. 5**). No significant modulation of the *TLP-5*, *PR-2*, *PR-4*, *GLP-7*, *OSM-1*, *TLP-4*, *CHI*, *NAC* or *GLP-2* genes was observed in whole leaves, microdissected stomata or surrounding cells of uninoculated and distal-inoculated samples.

The *TLP-5* gene was induced by *P. viticola* in local-inoculated samples, with comparable expression levels in whole fixed leaves, microdissected stomata and surrounding cells (**Fig. 5A**). The expression levels of the *PR-2*, *PR-4* and *GLP-7* genes were induced by *P. viticola* in local-inoculated samples (**Fig. 5B–D**). The expression levels of these genes in local-inoculated samples were greater in microdissected stomata

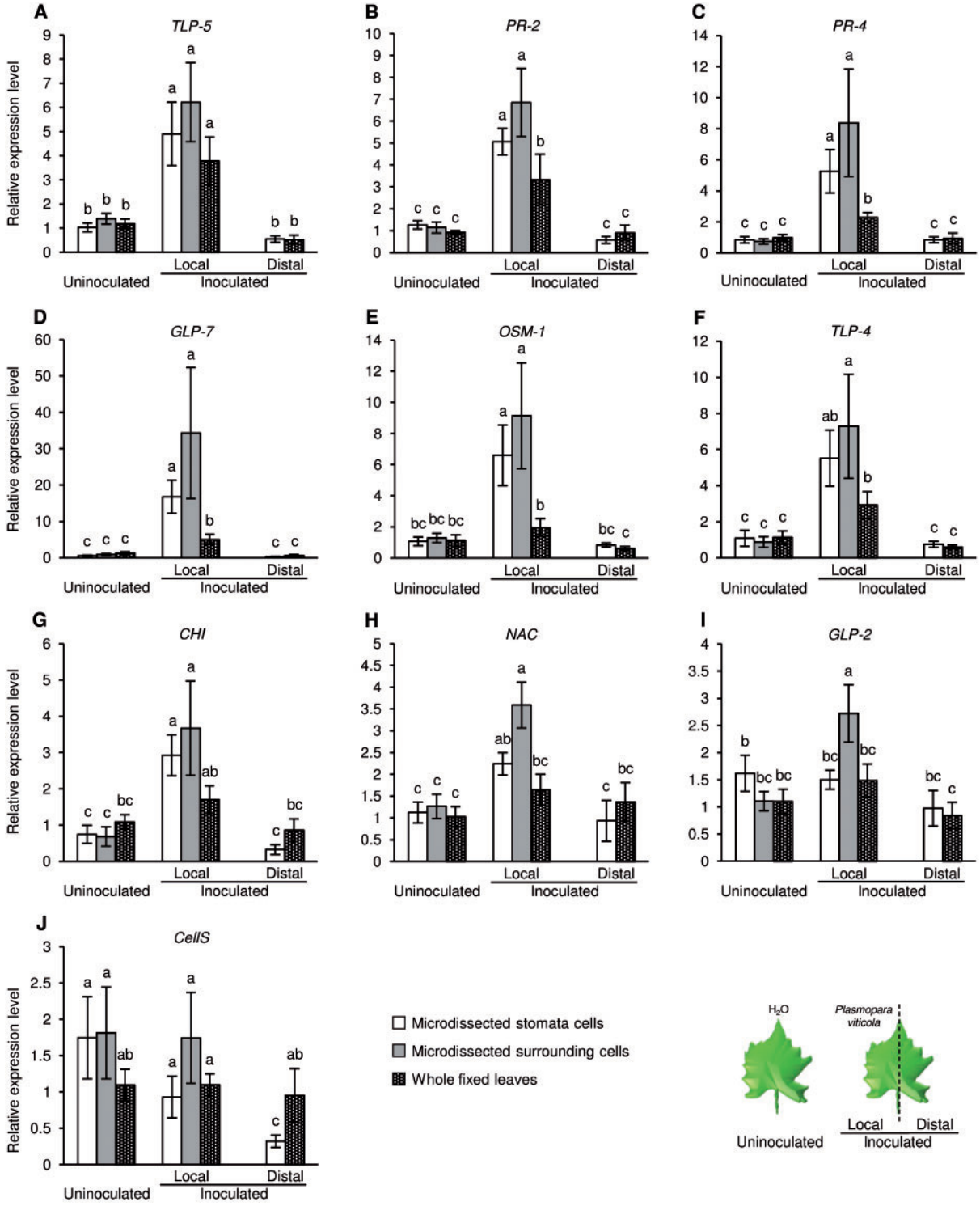

Fig. 5 Gene expression analysis of microdissected cells and whole fixed leaves of in vitro grown grapevines. Relative expression levels of genes encoding (A) thaumatin-like protein 5 (*TLP-5*), (B) pathogenesis-related protein 2 (*PR-2*), (C) pathogenesis-related protein 4 (*PR-4*), (D) germin-like protein 7 (*GLP-7*), (E) osmotin 1 (*OSM-1*), (F) thaumatin-like protein 4 (*TLP-4*), (G) chalcone isomerase (*CHI*), (H) NAC transcription factor (*NAC*), (I) germin-like protein 2 (*GLP-2*) and (J) cellulose synthase (*CellS*) were assessed by real-time PCR analysis. Leaves were treated with water (uninoculated) or inoculated with *Plasmopara viticola* (inoculated) under sterile conditions and fixed in 100% acetone at 24 hpi. Gene expression analysis was carried out on microdissected stomata cells, microdissected surrounding cells and in whole fixed leaves of uninoculated samples and inoculated (local-inoculated) or uninoculated areas of left-side inoculated leaves (distal-inoculated). Relative expression levels (fold change) were calculated using grapevine *Actin* as the constitutive gene for normalization, and data were calibrated on whole fixed leaves of the uninoculated sample. An *F*-test revealed non-significant differences between experiments (*P*-values ranged from 0.1 to 0.9 for the genes tested) and data from the two experiments were pooled. Mean levels of relative expression and SEs from 6–8 replicates pooled from two experiments are presented for each sample. For each gene, different letters indicate significant differences according to Fisher's test ($\alpha = 0.05$).

cells (5.1-, 5.3- and 16.8-fold for *PR-2*, *PR-4* and *GLP-7*, respectively) and surrounding cells (6.8-, 8.4- and 34.3-fold for *PR-2*, *PR-4* and *GLP-7* respectively) than in whole fixed leaves (3.3-, 2.3- and 5.0-fold for *PR-2*, *PR-4* and *GLP-7*, respectively).

The *OSM-1* gene showed similar expression profiles to *PR-2*, *PR-4* and *GLP-7* in microdissected cells of local-inoculated samples (6.6- and 9.1-fold induction in microdissected stomata and surrounding cells, respectively). However, the *OSM-1* expression of whole fixed leaves was not significantly affected by *P. viticola* in local-inoculated samples as compared with uninoculated samples (**Fig. 5E**). The expression level of *TLP-4* was significantly induced by *P. viticola* in local-inoculated samples (2.9-, 5.5- and 7.3-fold in whole fixed leaves, microdissected stomata and surrounding cells, respectively), and it was significantly greater in microdissected surrounding cells than in whole fixed leaves (**Fig. 5F**).

The expression of the *CHI* (**Fig. 5G**) and *NAC* (**Fig. 5H**) genes was significantly induced by *P. viticola* in microdissected stomata (2.9- and 2.2-fold, respectively) and surrounding cells (3.7- and 3.6-fold, respectively), but not in whole fixed leaves of local-inoculated samples. The *GLP-2* gene was induced (2.7-fold) exclusively in surrounding cells of local-inoculated samples (**Fig. 5I**). The expression levels of *NAC* and *GLP-2* genes were significantly greater in microdissected surrounding cells than in whole fixed leaves, while the expression level of *CHI* was not.

The expression level of *CellS* was not affected by *P. viticola* in whole fixed leaves and microdissected cells of uninoculated, local- and distal-inoculated samples. Only a slight decrease of *CellS* expression was observed in microdissected stomata of distal-inoculated samples (**Fig. 5J**).

Discussion

Transcriptional reprogramming is a crucial part of the interaction of biotrophic pathogens with host plants. However, gene expression analyses carried out to date have been done at a global level using whole leaves (Kortekamp 2006, Polesani et al. 2008, Legay et al. 2010, Polesani et al. 2010, Wu et al. 2010, Gamm et al. 2011, Perazzolli et al. 2012, Li et al. 2015, Merz et al. 2015), while only a small fraction of leaf cells was in contact with the pathogen at early stages of the infection. Therefore, many key transcriptional events that occurred exclusively in host cells in direct contact with the pathogen might have been diluted or masked by the greater abundance of non-infected cells when analyzing whole-organ samples (Coker et al. 2015). In this study, we characterized early grapevine responses to downy mildew with spatial resolution in sites with *P. viticola* infection, in surrounding cells and in distal leaf areas not infected by the pathogen, by specifically selecting groups of cells with an optimized LMD protocol. As sample fixation depends on the plant species (Nelson et al. 2006), we compared two protocols, and grapevine leaf fixation in 100% acetone proved to be the best option for preserving histological integrity and RNA quality. The effectiveness of the fixation protocol was further confirmed through comparable gene expression levels in whole fixed and whole fresh leaves after *P. viticola* inoculation for all the genes tested. Fixed samples were directly subjected to LMD without specimen sectioning by a microtome, saving time for paraffin embedding (Day et al. 2006), or by a cryotome, reducing the risk of morphological damage from ice crystals (Balestrini and Bonfante 2008). In comparison with fluorescence-activated cell sorting recently developed for *Arabidopsis thaliana* (Coker et al. 2015), our LMD method can be used at early time points after inoculation and allows morphology-based cell isolation, with no requirement for protoplast production or specific transgenic plants expressing infection markers. Because leaves of grown plants were used under axenic conditions, we could exclude interference from potential contaminant microorganisms (Barlass et al. 1986, Dai et al. 1995). Samples were collected when the formation of encysted zoospores, substomatal vesicles and primary hyphae established the first intimate contact between pathogen and host cells (Unger et al. 2007, Díez-Navajas et al. 2008, Polesani et al. 2010, Liu et al. 2015). The proportion of infected stomata was in agreement with previous observations (Yu et al. 2012), and the presence of *P. viticola* at 24 hpi was confirmed by amplification of the *P. viticola LSU* gene in whole leaves and microdissected stomata cells of local-inoculated samples. *Plasmopara viticola* was also detected in microdissected surrounding cells. Primary hyphae reached up to 40 μm in length at 24 hpi (Unger et al. 2007), and an area of 2,000 μm^2 (radius of about 25 μm) was collected for stomata cells, indicating that some primary hyphae may have been sampled from the surrounding areas.

The expression levels of 10 grapevine genes known to be markers for defense responses (Godfrey et al. 2007, Polesani et al. 2008, Legay et al. 2010, Perazzolli et al. 2011, Perazzolli et al. 2012, Santi et al. 2013) were examined in microdissected stomata and surrounding cells, and were compared with those of whole leaves. In particular, seven defense-related genes (*PR-2*, *PR-4*, *OSM-1*, *TLP-4*, *TLP-5*, *GLP-7* and *GLP-2*), one transcription factor (*NAC*) and two genes related to secondary metabolic processes (*CHI* and *CellS*) were analyzed. As reported in previous studies (Godfrey et al. 2007, Perazzolli et al. 2011, Perazzolli et al. 2012), expression of the *PR-2*, *PR-4*, *OSM-1* and *GLP-7* genes was induced by *P. viticola* in whole leaves. Conversely, the absence of the *P. viticola*-dependent induction of *GLP-2* (Godfrey et al. 2007), *CHI* (Legay et al. 2010), *NAC* and *CellS* (Polesani et al. 2008) in whole leaves may be related to the use of different plant genotypes, time points and inoculation methods. Indeed, the *CellS* gene was induced by *P. viticola* in Riesling (Polesani et al. 2008) and not in Pinot noir plants, in agreement with the different transcriptional response of susceptible genotypes to *P. viticola* (Banani et al. 2014). The induction of *GLP-2* and *NAC* was previously observed at late stages of infection (Godfrey et al. 2007, Polesani et al. 2008), suggesting that heavy *P. viticola* invasion of grapevine tissues is required to detect the modulation of these genes by whole-leaf analyses. *CHI* expression was induced in whole leaves by spray inoculation of *P. viticola* on greenhouse-grown plants (Legay et al. 2010), while its expression change after in vitro inoculation was detected only in microdissected cells.

The use of spray inoculation and greenhouse conditions could also explain the greater magnitude of *PR-4* and *OSM-1* modulation in Perazzolli et al. (2011, 2012) than in our experiments.

The extent of gene modulation was generally greater in microdissected cells than in whole leaves, demonstrating that the analysis of selected tissue portions dramatically increases sensitivity for detecting transcriptional changes. Furthermore, the dilution effect of whole-leaf analysis totally masked the modulation of the *GLP-2*, *NAC* and *CHI* genes, which was significantly affected by *P. viticola* in the microdissected cells. The expression levels of the *PR-2*, *PR-4*, *OSM-1*, *GLP-2*, *GLP-7*, *TLP-4* and *NAC* genes were greater in microdissected stomata and surrounding cells than in whole leaves, indicating site-specific regulation of grapevine response to *P. viticola*. Specifically, the gene expression profiles of defense-related genes (*PR-2*, *PR-4* and *OSM-1*) suggested that the attempted defense reaction of susceptible grapevine (Polesani et al. 2010) was mainly activated corresponding to the infection sites at 24 hpi. The specific expression profiles of the *GLP* family members in stomata cells and surrounding areas indicated different roles in grapevine response. For example, *GLP-2* was modulated by *P. viticola* exclusively in microdissected surrounding cells. *GLP-2* is closely related to the *PsGER1* gene from *Pisum sativa* (Godfrey et al. 2007), which encodes a receptor of bacterial cell surface proteins (Swart et al. 1994), and it could be responsible for the *P. viticola* recognition around the infection sites. Conversely, the *GLP-7* gene was induced in both stomata and surrounding cells and is related to the epidermal-specific *Hordeum vulgare* protein HvGER4 (Godfrey et al. 2007), which is involved in the formation of papilla in response to powdery mildew (Wei et al. 1998), and it is possibly implicated in the defense reaction of cells in contact with the pathogen. The *TLP* genes showed specific modulation patterns in microdissected cells, possibly due to the spatial regulation following *P. viticola* inoculation, as reported in response to stolbur infection (Santi et al. 2013). *TLP* genes belong to the *PR-5* family, which is associated with antifungal activity (Monteiro et al. 2003), and the *TLP-5* gene was expressed at comparable levels in whole leaves and microdissected cells, indicating a global response to *P. viticola* infection. Conversely, a more localized role for *TLP-4* in the restriction of pathogen development at the infection sites was suggested by greater expression levels in microdissected surrounding cells than in whole leaves. Likewise, the *P. viticola*-dependent induction of *CHI* and *NAC* was detected exclusively in microdissected stomata and surrounding cells and not in whole leaves. In particular, *CHI* encodes an enzyme responsible for the production of phytoalexins (Legay et al. 2010), indicating local accumulation of key defense molecules against downy mildew (Slaughter et al. 2008) near the infection sites. The expression profiles of the *NAC* transcription factor in microdissected cells correlated well with those of defense-related genes, but further investigations are required to prove its involvement in site-specific regulation of grapevine responses to *P. viticola*. The modulation of *PR-2*, *PR-4*, *GLP-7*, *OSM-1*, *TLP-4*, *TLP-5*, *CHI* and *NAC* was comparable in microdissected stomata and in surrounding areas, suggesting that short-distance signals are released from infected stomata to adjacent cells. Indeed, Guan et al. (2014) reported that stomatal guard cells act as gatekeepers of *P. viticola* infection and that a cytoskeletal-based response is activated in neighboring cells. However, none of the tested genes, including the *PR-4* gene, which is a marker of jasmonic acid (JA) signaling (Hamiduzzaman et al. 2005), was modulated by *P. viticola* in uninoculated areas of left-side inoculated leaves at 24 hpi. Therefore, these expression profiles indicate that defense signals and the JA-mediated response to *P. viticola* (Polesani et al. 2010) were not yet diffused at long distances from the infection site at 24 hpi.

Conclusions

The LMD protocol used in this work permitted transcriptional analysis of grapevine response to *P. viticola* with spatial resolution by specific isolation of selected leaf cells. In this study, we identified site-specific regulation of the transcriptional response at *P. viticola* infection sites. The gene modulation was generally comparable in microdissected stomata cells and in surrounding areas, indicating that defense signals rapidly stimulated grapevine responses in leaf cells near to the infection sites. However, long-distance signals were not diffused at early stages of infection and genes were not modulated in distal-inoculated tissues. The LMD technique highlighted greater magnitudes of gene modulation in microdissected cells than in whole leaves, and identified transcriptional changes that were totally masked at the whole-tissue level. Therefore, our LMD method increases the sensitivity of gene expression analyses and it could lead to the identification of novel genes involved in the local reaction of grapevine leaves to *P. viticola* in future transcriptomic studies.

Materials and Methods

Plant material and *Plasmopara viticola* inoculum

Plants of the susceptible *V. vinifera* cv. Pinot noir ENTAV115 were grown in vitro in 75 ml glass tubes for plant tissue culture (SciLabware Limited) on 20 ml of half-strength Murashige and Skoog (MS) basal medium pH 5.5 ± 0.1 augmented with 0.6 mg l^{-1} (w/v) thiamine, 100 mg l^{-1} (w/v) myo-inositol, 30 g l^{-1} (w/v) sucrose and 6 g l^{-1} (w/v) agar. Plants were grown for 2 months in a growth chamber at 23 ± 1°C with a photoperiod of 16 h light.

A *P. viticola* population was collected from an untreated vineyard in northern Italy (Trentino region) in 2014 and maintained by subsequent inoculations on Pinot noir plants under greenhouse conditions at 25 ± 1°C, with a photoperiod of 16 h light and a relative humidity of 70 ± 10%, as described by Perazzolli et al. (2011). To obtain a sterile inoculum suspension of *P. viticola* sporangia, infected leaves showing early downy mildew symptoms (oil spots) were collected and washed in a 1% sodium hypochlorite solution for 10 min under orbital shaking at 80 r.p.m. (Márquez et al. 2007). Surface-sterilized leaves were washed three times for 5 min each in sterile distilled water under orbital shaking at 80 r.p.m. Leaves were transferred with the abaxial side uppermost onto sterile moist filter paper (three foils) in autoclaved glass Petri dishes (175 mm diameter), and incubated overnight in the dark at room temperature to allow downy mildew sporulation (Algarra Alarcon et al. 2015). Leaves bearing freshly sporulating lesions were transferred to a 50 ml sterile tube and gently washed with 5 ml of cold (4°C) sterile distilled water. The inoculum suspension was filtered with a sterile fine net and the concentration was adjusted to 4×10^4 sporangia ml^{-1} by counting with a hemocytometer under a light microscope.

Inoculation of grapevine leaves with *Plasmopara viticola* and sample collection

Leaves from 12 in vitro grown plants were detached and placed randomly on Petri dishes (92 mm diameter) containing 25 ml of 0.7 g l^{-1} water agar (MicroAgar; Duchefa Biochemie). For each dish, 2–3 leaves were placed abaxial side uppermost. Five leaves were inoculated by applying six to eight 2 µl drops of the fresh *P. viticola* sporangia suspension to the abaxial surface of each leaf under sterile conditions (local-inoculated sample). In order to investigate the distal activation of grapevine defense, three leaves were inoculated exclusively in the area to the left of the primary vein by applying 3–4 drops of the sporangia suspension under sterile conditions (left-side inoculated leaf), and the area to the right of the primary vein was not inoculated with the pathogen (distal-inoculated sample). As control, the abaxial surfaces of six grapevine leaves were treated with 6–8 drops of sterile distilled water under sterile conditions (uninoculated sample). Leaves were incubated overnight in the dark at 25 ± 1°C to allow *P. viticola* infection, then dried using a sterile filter paper under sterile conditions, and samples were collected at 24 hpi from uninoculated and inoculated samples. This time point was chosen because it is associated with the formation of the substomatal vesicle and the primary hyphae of *P. viticola* (Unger et al. 2007, Godard et al. 2009), and with up-regulation of defense-related genes (Hamiduzzaman et al. 2005, Polesani et al. 2008, Trouvelot et al. 2008, Perazzolli et al. 2011, 2012).

Five replicates (each composed of 6–8 leaves for uninoculated and inoculated samples, respectively) were used for each sample, and the experiment was carried out twice. Four types of analysis were carried out for each replicate: (i) three half leaves of uninoculated and local-inoculated samples were immediately frozen in liquid nitrogen and stored at −80°C for gene expression analysis of the whole fresh leaves; (ii) three half leaves of uninoculated, local-inoculated and distal-inoculated samples were subjected to the fixation protocol for LMD; (iii) two half leaves of uninoculated and local-inoculated samples were stained with aniline blue to assess the proportion of infected stomata; and (iv) two leaves of uninoculated and local-inoculated samples were incubated on agar plates for 7 d in a growth chamber at 24 ± 1°C and a photoperiod of 16 h light to evaluate the development of downy mildew symptoms.

Aniline blue staining of *Plasmopara viticola* and assessment of the percentage of infected stomata

In order to observe *P. viticola* structures, leaf samples were stained with the KOH–aniline blue fluorescence method (Díez-Navajas et al. 2007) with slight modifications. Leaves were clarified in 1 M KOH at 95°C for 15 min and washed with fresh 1 M KOH by incubation at room temperature for 15 min. Samples were washed three times in distilled water for 15 min each, and incubated in the staining solution (0.05% aniline blue in 0.067 M K_2HPO_4 at pH 8.0) for 15 min. Leaf samples were transferred to microscope slides with the lower side up, and microscopic observations were carried out with an LMD7000 instrument (Leica Microsystems) using an LMD filter (BP filter 380–420 nm excitation, 415 dichroic mirror, and BP 445–485 nm emission). For each half leaf, 10 areas of 1 mm^2 each were randomly selected, and the number of stomata with encysted zoospores or with a substomatal vesicle with primary hyphae and haustoria (infected stomata) was visually assessed under the fluorescent microscope and expressed as a percentage of the total number of stomata.

Assessment of downy mildew severity

Disease severity was assessed visually at 7 d post-inoculation with *P. viticola* as a percentage of infected leaf area on the abaxial leaf surface according to standard guidelines of the European and Mediterranean Plant Protection Organization (EPPO 2001). For each leaf, disease severity was expressed as a percentage of abaxial leaf area covered by white sporulation of *P. viticola* in relation to total leaf area.

Downy mildew severity was also assessed as the number of *P. viticola* sporangia per gram of leaf fresh weight. The fresh weight of all leaves of each replicate was determined and leaves were then washed by gently vortexing in 1 ml of sterile water for 1 min to collect *P. viticola* sporangia. The concentration of sporangia per ml was determined by counting with a hemocytometer under a light microscope and it was converted to number of sporangia per gram of fresh weight of grapevine leaves (sporangia g^{-1}).

RNA extraction and cDNA synthesis from whole fresh leaves

For RNA extraction from whole fresh leaves, samples were homogenized by two grinding cycles at 25 Hz for 30 s in a mixer mill (Retsch Technology GmbH). Total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich), quantified by NanoDrop 8000 (Thermo Fisher Scientific), and its quality was checked by agarose gel electrophoresis.

The total RNA of whole fresh leaves was treated with DNase I (Invitrogen, Life Technologies), and the first-strand cDNA was synthesized from 1 µg of total RNA using SuperScript III (Invitrogen, Life Technologies) and oligo(dT) primers. cDNA obtained from whole fresh leaves was diluted 10 times for real-time PCR analysis.

Tissue fixation for laser microdissection

Leaf samples were cut into small pieces (about 0.5 cm^2) with a sharp razor blade in ice-cold fixative solution, and two different fixative solutions were tested: cold ethanol–acetic acid (3 : 1) solution and 100% acetone. Samples were immediately transferred in 3 ml of cold (0°C) fixative solution in a 20 ml plastic syringe (Artsana S.p.a). A vacuum was applied three times for 1 min each and rapidly released to allow fast penetration of the fixative solution into the leaf tissues (Vitha and Osteryoung 2011). Leaf samples were transferred into 30 ml of cold (0°C) fixative solution in a 50 ml sterile tube, incubated in ice for 10 min and gently swirled on a rotator (20 r.p.m.) at 4°C overnight. Fixed samples were stored in the fixative solution at −20°C for up to a maximum of 14 d prior to LMD. Preliminary experiments revealed that high-quality RNA can be extracted from samples stored for 5, 11 or 14 d in the fixative solution, but not those stored for 18 or 21 d.

Laser microdissection of grapevine leaves

The fixative solution was removed by decanting, and fixed leaves were washed briefly in 10 ml of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, pH 7.3 in RNase-free water). Fixed leaves were laid on PEN slides (Leica Microsystems) with the abaxial side up, dried at room temperature and subjected to LMD. LMD was carried out with an LMD7000 instrument (Leica Microsystems) using the ×40 objective and laser parameters power 48 mW, aperture 1, speed 5 ms, in order to isolate specifically stomata and the surrounding cells. Microdissected stomata and surrounding cells were collected from uninoculated and local-inoculated samples, and stomata cells were also microdissected from distal-inoculated samples. Microdissected cells were directly collected in a 0.5 ml RNase-free PCR tube cap containing 30 µl of extraction buffer (PicoPure RNA Isolation kit; Arcturus) that was located in the collection vessel directly under the microscope slides. In a preliminary experiment, different numbers of stomata (1, 10, 50 100, 300 and 500) were collected from whole fixed leaves, and samples of at least 300 stomata, corresponding to >0.7 mm^2, showed good quantity and quality of extracted RNA. In order to limit the risk of RNA degradation and to prevent sample heating by the microscope lamp, each replicate was collected in two PCR caps (150 microdissected cells each) in <1.5 h.

After LMD, PCR tubes were closed and centrifuged at 3,000×*g* for 1 min to collect microdissected cells at the bottom. To transfer all collected cells to the tube bottom, the cap was washed with 20 µl of lysis buffer, and the tube was centrifuged at 3,000×*g* for 1 min. In order to verify the RNA quality at the end of the LMD and to analyze the gene expression levels of whole fixed leaves, leaf tissues that remained on the microscope slides were scraped off from the PEN slide and collected in 2 ml RNase-free PCR tubes. Samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

RNA extraction and cDNA synthesis from microdissected samples

RNA was extracted from microdissected cells using a PicoPure RNA Isolation kit and eluted in 14 µl of RNase-free water. RNA was extracted from whole fixed leaves using a PicoPure RNA Isolation kit with 200 µl of extraction buffer, 200 µl

of 70% ethanol and 25 µl of RNase-free water. For each replicate, the two PCR tubes obtained by LMD were pooled after the lysis step. An RNase-free DNase set kit (Qiagen) was used during RNA extraction in order to remove genomic DNA.

The quality of RNA extracted from the microdissected cells and whole fixed leaves was assessed with a 2100 Bioanalyzer (Agilent Technologies) using an RNA 6000 Pico Assay kit (Agilent Technologies) and an RNA 6000 Nano Assay kit (Agilent Technologies), respectively.

For each replicate, 10 µl (0.3–2.5 ng μl^{-1}) of total RNA extracted from microdissected cells and 400 ng of total RNA extracted from whole fixed leaves were subjected to one round of RNA amplification using the MessageAmp II aRNA Amplification kit (Ambion, Life Technologies). The reaction was carried out at 37°C for 14 and 8 h for RNA of microdissected cells and whole fixed leaves, respectively. The amplified RNA (aRNA) was purified according to the manufacturer's instructions, eluted in 100 µl of RNase-free water and quantified with a NanoDrop ND-8000 (Thermo Fisher Scientific). The aRNA from microdissected cells was concentrated to approximately 30 µl in a vacuum centrifuge (Eppendorf). For both microdissected cells and whole fixed leaves, 14 µl of aRNA (about 15–45 ng μl^{-1}) were reverse-transcribed using the SuperScript VILO (Invitrogen, Life Technologies) in a total volume of 20 µl. The cDNA was diluted 10 and 20 times for the detection of the *P. viticola* and grapevine genes by real-time PCR analysis, respectively.

Quality control of cDNA by PCR analysis

In order to verify the synthesis of cDNA and the absence of DNA contamination, PCR amplification was carried out from 1 µl of cDNA, using intron-spanning primers designed on a grapevine *Actin* gene (VIT_204s0044g00580, *Act_PCR*_For: 5′-TGACAATGGAACTGGAATGGT-3′; *Act_PCR*_Rev: 5′-ATCAG GCAGCTCTATAGTTCTTC-3′). PCRs were carried out using DreamTaq DNA Polymerase (Fermentas) with a T-Professional Thermocycler (Biometra), using the following PCR conditions: 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 60°C for 30 s and 68°C for 1 min. Amplification of the expected fragment without DNA contamination was verified by agarose gel electrophoresis.

Quantitative real-time PCR analysis

Quantitative real-time PCRs were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Life Technologies) and specific primers for markers of grapevine response to downy mildew (**Table 1**) using the Light Cycler 480 (Roche Diagnostics). Reactions were set with two initial steps at 55°C for 10 min and at 95°C for 2 min, followed by 50 and 40 cycles at 95°C for 15 s and 60°C for 1 min for the grapevine and *P. viticola* genes, respectively. Each sample was examined in three technical replicates, and dissociation curves were analyzed to verify the specificity of each amplification reaction. Cycle threshold (Ct) values were obtained by second derivative calculation using the Light Cycler 480 1.5.1.62 SP2 software (Roche). Reaction efficiencies were calculated with the LinRegPCR 11.1 software (Ruijter et al. 2009), and the relative expression level of each grapevine gene was calculated according to the Pfaffl equation (Pfaffl 2001) using uninoculated samples for calibration. Grapevine *Actin* (VIT_212s0178g00200) was used as the constitutive gene for normalization because its expression was not significantly affected by *P. viticola* inoculation (Polesani et al. 2010, Perazzolli et al. 2011). Pathogen development in the leaf samples was estimated from the expression of the *LSU* gene of *P. viticola* (KM279688) (Gindro et al. 2014), and normalized to the transcription level of the grapevine *Actin* gene (Casagrande et al. 2011).

Primer design

Primer pairs for the real-time PCR analysis of grapevine germin-like proteins (*GLP-2* and *GLP-7*) and *P. viticola LSU* genes, and for the PCR analysis of the grapevine *Actin* gene were designed using the Primer3 software V. 4.0 (http://primer3.sourceforge.net) and analyzed with the Oligo Analyzer V.1.0.2 (www.bio.net/bionet/mm/bio-soft/2001-September/023431.html).

Statistical analysis

Experiments were repeated and data were analyzed using Statistica 9 software (StatSoft). An *F*-test was used to demonstrate non-significant differences between two experiments ($P > 0.05$), and data from the two experimental repetitions were pooled. Fold change values of gene expression analysis were transformed using the equation $y = \log_{10}(1 + x)$ (Casagrande et al. 2011). After validation of normal distribution (K–S test, $\alpha < 0.05$) and variance homogeneity of the data (Levene's test, $\alpha < 0.05$), analysis of variance (ANOVA) was carried out and Fisher's test ($\alpha = 0.05$) was applied to detect significant differences.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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