Powdery mildew responsive genes of resistant grapevine cultivar 'Regent'

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Summary

The ascomycete Erysiphe necator causes powdery mildew disease of grapevine, a disastrous infection which is commonly defeated with multiple fungicide applications in viticulture. Breeding for natural resistance of quality grapes (Vitis vinifera) is thus a major aim of current efforts. The cultivar 'Regent' is resistant to powdery mildew due to an introgression from an American Vitis sp. resistance donor. To identify key regulatory elements in defense responses of 'Regent' we performed transcript analyses after challenging with E. necator inoculation in comparison with a susceptible grapevine. A set of genes selected from preliminary microarray hybridization results were investigated by RT-qPCR. The data indicate an important role of transcription factors MYB15, WRKY75, WRKY33, WRKY7, ethylene responsive transcription factors ERF2 and ERF5 as well as a CZF1/ZFAR transcription factor in regulating the early defense when the fungus starts the interaction with its host by the formation of haustoria.

Key words: Erysiphe necator; gene induction; transcription factors; ERF; MYB; WRKY.

Introduction

Grapevine (Vitis vinifera L.) provides the basis of viticulture since ancient times. However, its prosperity is endangered by pathogens like Erysiphe necator Schwein. (syn. Uncinula necator; anamorph Oidium tuckeri Berk.), the causal agent of powdery mildew. This fungus was accidentally introduced to Europe in the 19th century from North America. Encountering a naïve host, no resistant cultivars of Vitis vinifera were available and tremendous amount of fungicides are applied to ensure a good quality harvest from current elite cultivars (Eurostat 2007). E. necator is an ascomycete that infects its host predominantly by vegetatively produced conidia. The mycelium grows epiphytically on the host tissue, produces appressoria that poke holes into the cuticula and invades the underlying epidermal cells with haustoria to retrieve nutrients from the host cell (HEINTZ and BLAICH 1990). Depending on the climatic conditions, but usually after only a few warm summer days, the fungus has developed sufficiently to propagate by production of conidiophores on the leaf surface that release terminally constricted conidia. These are easily broken-off and dispersed by air current to start new infections on suitable host tissue. E. necator is an obligate biofrophic fungus and specific for grapevine (GADOURY et al. 2011).

Breeding for natural resistance in combination with best quality is a major aim of programs to improve grapevine cultivars (EIBACH et al. 2007). It relies on the introgression of resistance traits identified mostly in American and Asian wild Vitis sp. that are inter-fertile with V. vinifera. The closely related American Muscadina rotundifolia has also been exploited as a donor of potent resistance genes (BARKER et al. 2005, RIAZ et al. 2011). During the last two decades, due to the development of genetic mapping and QTL analysis methods, resistance loci in several experimental populations have been tagged and linked to molecular markers (references and loci as compiled in www.vivc.de, data on breeding and genetics). These are applied in marker-assisted selection for breeding. They enable the tracking of combinations of several loci of diverse origins. The aim is to generate durable resistance by pyramiding resistance genes. Up to now, 13 different Ren (resistance to Erysiphe necator) and Run (resistance to Uncinula necator) loci have been detected and described. They typically encode small gene families of NBS-LRR (nucleotide binding site – leucine rich repeat) type resistance gene analogs thought to function as receptors that interact directly or indirectly with pathogen strain-specific effectors (EITAS and DANGL 2010). This interaction is termed effector-triggered immunity (ETI). It initiates a signaling cascade which leads to transcriptional re-programming in the host plant and expression of plant defense genes. In the case of the M. rotundifolia locus Run1 it was shown that a gene from a TIR (Toll-Interleukin-receptor domain)-NBS-LRR gene cluster on chromosome 12 mediates resistance (FEECHIAN et al. 2013). In general, there is rather limited knowledge about the resistance mechanisms encoded in the various Ren/Run loci (QIU et al. 2015). In addition to ETI, plants possess PTI (pathogen triggered immunity) as a basal defense mechanism. In this reaction ubiquitous signals of a pathogen attack (so-called “pathogen-associated molecular patterns”, PAMPs, e.g. chitin from fungi) are recognized by plant receptor kinases that transmit the sensing through a
phosphorylation cascade to the transcription machinery of the host cell. This causes re-organization of the transcriptional program and expression of defense-related genes. The pathways of ETI and PTI partially overlap on a molecular level. Pathogen effectors are considered to be components that developed to suppress PTI. Resistant plants evolved ETI to counter this effect (for review see Muthamilarasan and Prasad 2013, Eitas and Dangi 2010).

In the frame of breeding research we identified the resistance locus Ren3 from the powdery mildew resistant cultivar 'Regent' (Fischer et al. 2004) and tagged it with markers useful for breeder's selection (Akkurt et al. 2007, Welter et al. 2007). The Ren3 locus was recently detailed and found to be flanked by locus Ren9. Ren3/9 carriers exhibit a hypersensitive response to E. necator clearly evident at 5 dpi (days post inoculation). Powdery mildew resistance of 'Regent' relies on a "post-invasion" mechanism that restricts pathogen development and finally impairs the formation of conidia (Zendler et al. 2017). Molecular analysis of this locus is under way. However, besides the analysis of positional candidate genes found in QTL regions of the grapevine genome, there is an alternative strategy to detect resistance-associated genes by differential gene expression studies. This approach yields information that helps to reveal the complex regulatory pathways operating in grapevine immunity. It identifies key regulatory components that could possibly be combined with QTL regions to optimize the defense response in newly-bred cultivars. So we started to characterize the transcriptional changes of the Ren3/9 carrier 'Regent' elicited by E. necator inoculation. Data were compared to susceptible grapevines. To determine the best time point for this investigation the infection progress was monitored by microscopy during the early phases after experimental inoculation. RNA prepared from the point of the newly established host/pathogen interaction was then subjected to a preliminary microarray analysis followed by quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) to validate a selected set of genes. The focus was laid on transcription factor genes as key elements of gene regulation.

**Material and Methods**

**Experimental inoculations:** Young leaves from susceptible grapevine plants ('Müller-Thurgau') grown in the greenhouse with natural infections at the stage of sporulation were used as a source of powdery mildew conidia. Since greenhouse infections occur spontaneously and conidia were collected from several plants, the inoculum rather represents a mixture of E. necator strains than a specific strain. Plantlets of 'Regent' and susceptible controls ('Lemberger') were studied by epifluorescence microscopy (Zeiss Axiovision 4 microscope with filterset 05 (Exitation 405 nm, Emission 420-450 nm), 100 conidia per leaf were counted (1993) with modifications.

**Gene expression studies were performed with plantlets of cultivars 'Regent' and 'Chardonnay' grown in Weck® glasses. Three plants were raised per 580 mL container on 0.5x MS medium. The plants were inoculated at the 6-8 leaf stage. Infested source leaves were sliced into small pieces under sterile conditions. The spores from those leaf pieces were allowed to drop onto the leaves of the test plants. Three to five leaves per plant from at least five glass containers per cultivar (15 plants in total) per replication were inoculated by allowing conidia from the infested source leaf to drop onto their surface. The Petri dishes were closed, protected from desiccation by wrapping in plastic wrap and incubated at 25 °C under a 12 h light/12 h dark regime. Sampling was done at 0, 6, 10 and 24 h post inoculation (hpi). The leaves were de-stained and preserved in 70 % Ethanol (Berkel AHK, Ludwigshafen, Germany) until analysis. Three leaves were investigated per time point and cultivar. This experiment was repeated three times.

To obtain inoculum for subsequent gene expression studies on 'Regent' the conidia of E. necator were propagated through at least five cycles of asexual reproduction. Surface-sterilized detached leaves of 'Müller Thurgau' were placed adaxial side up in 580ml Weck® glass containers (Weck, Wehr, Germany) containing 1% agar in water. Every 14 d spores from the powdery mildew colonies were transferred to newly surface-sterilized detached leaves.

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**Microscopy:** The first phases of conidia behaviour on 'Regent' as compared to a susceptible grapevine cultivar ('Lemberger') were studied by epifluorescence microscopy (Figure, a). The samples were watered and stained with a few drops of "Direct Yellow 96" (Sigma Aldrich, Munich, Germany; 0.05 % in H2O), 100 conidia per leaf were counted and scored according to their developmental stages (non-germinated, germ tube appeared, appressorium formed) using a Zeiss AxioVision 4 microscope with filterset 05 (Exitation 395-440 nm, Emission 470 nm). The data (Figure, b) were processed using ANOVA (XLSTAT) to check for normal distribution at a significance level of α = 5 %.

**RNA extraction:** Total RNA was isolated following the protocol of Chang et al. (1993) with modifications. The leaves from each treatment were bulked and ground with liquid nitrogen using a mortar and a pestle. About 100 mg of leaf powder was mixed with 800 mL of 65 °C pre-warmed extraction buffer (2 % CTAB (Cetyltrimethylammonium bromide, Serva, Heidelberg, Germany), 2 % PVP (Polyvinylpyrrolidone, Sigma-Aldrich, Taufkirchen, Germany) K30, 100 mM Tris-HCl (pH 8.0; Roth, Karlsruhe, Germany), 25 mM EDTA (Ethylenediamine tetra-acetic acid; Merck, Darmstadt, Germany), 2.0 M NaCl (Roth), 0.5 g L-1 Spermidine (Serva) and 2 % β-mercaptoethanol (Sigma-Aldrich, Kremsmünster, Austria) under sterile conditions. The leaves were inoculated by allowing conidia from the infested source leaf to drop onto their surface. The Petri dishes were closed, protected from desiccation by wrapping in plastic wrap and incubated at 25 °C under a 12 h light/12 h dark regime. Sampling was done at 0, 6, 10 and 24 h post inoculation (hpi). The leaves were de-stained and preserved in 70 % Ethanol (Berkel AHK, Ludwigshafen, Germany) until analysis. Three leaves were investigated per time point and cultivar. This experiment was repeated three times.

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added just before use) and incubated for 10 min at 65 °C. An equal volume of Chloroform:Isoamyl alcohol (24:1; Roth) was added to the homogenate, mixed by inverting the tubes during 5 min. and the mixture centrifuged at 8,944 g for 10 min. at 4 °C. The inorganic phase was submitted to a second organic extraction. The supernatant was collected and 0.25x volume of 10 M Lithium chloride (Sigma-Aldrich) was added. The RNA was precipitated over night at 4 °C and pelleted by centrifugation at 20,937 g for 30 min at 4 °C. The RNA quality and quantity were monitored by denaturing 1.5 % agarose (Lonza, Basel, Switzerland)/1.0 % formaldehyde (Sigma-Aldrich) gel electrophoresis and UV-spectrophotometry. The RNA was treated with DNaseI (Qiagen, Hilden, Germany) and purified with RNeasy MinElute CleanUp kit (Qiagen) according to the supplier's instructions. RNA quality and quantity were re-checked by gel electrophoresis and spectrophotometry.

Primer design for RT-qPCR: A set of 26 powdery mildew-induced genes was analysed by quantitative Real Time PCR (RT-qPCR). Nucleotide sequences for primer design were retrieved from public databases. They were compared to the grape EST database (originally available at http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=grape and shifted to ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/Genomes/Vitis_vinifera/) and highly similar sequences (possible members of gene families) were studied in detail. The annotation compiled in CRIBI (http://genomes.cribi.unipd.it/grape/#) was used. Primers were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) (with settings Tm: 58-60 °C, amplicon length: 70-150 bases). The primer sequences are listed in the supplementary Table. All primers were tested for PCR efficiency and specificity. Melting curves of the amplification products confirmed the absence of any primer dimer or nonspecific amplification products. The PCR products were separated in 2 % agarose gels to confirm their sizes.

Quantitative Real-time PCR analysis: Two-step RT-qPCR was performed. At first 1 μg of total RNA was reverse-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. In the second step, the cDNA was amplified by qPCR. PCR reactions were performed in 96-well plates with an ABI PRISM® 7500 amplification and detection system using SYBR® Green as a fluorescent reporter dye. PCR reactions were done in mixtures of 25 μL, containing 12.5 μL Power SYBR® Green PCR Master Mix (Applied Biosystems), 150-300 nM of each primer and 25 ng cDNA of each sample. The thermal cycling conditions consisted of one initial polymerase activation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for one min. Raw fluorescence data were exported to Excel spreadsheets and used to estimate the PCR efficiency for each sample employing the LinRegPCR software V7.2 (RAMAKERS et al. 2003). The cycle threshold value (Ct) as a starting point and the raw fluorescence information of at least four PCR cycles were applied to estimate PCR efficiency by linear regression. In every case the correlation factor (R) was greater than 0.99. The Ct value was defined by the 7500 Fast System SDS Software (Applied Biosystems, Foster City, CA, USA) with manual baseline (3-15) and threshold (0.2) settings. A mean PCR efficiency including all samples of each gene was calculated. Together with the Ct values of target and control samples the mean PCR efficiency was used to calculate the relative gene expression (inoculated against
non-inoculated) employing REST 2005 software (V1.9.12) (Pfaffl et al. 2002). β-tubulin- and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes served as references (supplementary Table).

**Results and Discussion**

**Determination of time point for differential gene expression studies:** To isolate total RNA representative of the early host/pathogen interaction the progress of infestation after inoculation of resistant 'Regent' with powdery mildew conidia was investigated in comparison to susceptible 'Lemberger'. 'Regent' has a post-invasion resistance that becomes obvious after 5 d of infestation (Zendler et al. 2017). At 6, 10 and 24 hpi there was no difference in the interaction of the pathogen between the resistant, and the susceptible host. Conidia germinated on in vitro leaves of both host cultivars with the same success (Figure, a). They formed lobed and mature appressoria with a frequency of around 60 % at 10 hpi (Figure, b). At this stage they were starting to penetrate the host cell and a close interaction between pathogen and host was established. It can be presumed that defense reactions should be efficiently activated at this stage. For this reason, the time point of 10 hpi was chosen for analysis of the defense responses on molecular level.

**Differential gene expression analysis:** According to results from the inoculation study samples were collected at 10 hpi from inoculated and non-inoculated resistant 'Regent' and equally treated susceptible leaves ('Chardonnay'). Since no susceptible isogenic line of 'Regent' is available, a susceptible V. vinifera genotype suitable for in vitro propagation was employed for comparison. The RNA of these samples was extracted and preliminarily investigated by microarray analysis. Comparative hybridizations to the Array-Ready Oligo Set™ immobilized on glass chips (as described in Terrier et al. 2005) revealed around 70 genes up-induced at least 1.5-fold in 'Regent' and activated to significantly higher levels in 'Regent' as compared to 'Chardonnay' (data not shown). Further work focused on a subset of genes evaluated by RT-qPCR. Genes appearing activated particularly during the incompatible interaction were of major interest. As the aim was to gain insight into key regulatory pathways of successful defense, genes encoding transcription factors were selected. Genes encoding PR (pathogenesis-related) proteins and enzymes involved in the secondary pathway were also analyzed to investigate by microarray analysis. Comparative hybridizations to the Array-Ready Oligo Set™ immobilized on glass chips (as described in Terrier et al. 2005) revealed around 26 genes studied, 22 showed at least a two-fold transcriptional induction in 'Regent' challenged with powdery mildew. Genes encoding PR (pathogenesis-related) proteins and enzymes including salicylic acid (SA). It is well known that DAHP, EPSPS and PAL are transcriptionally activated by wound- and pathogen attack. COMT can act on caffeic acid to produce monolignols and precursors of lignin biosynthesis (Bowsheir et al. 2008). These may participate in the early defense reaction at the appressorial contact sites of the pathogen as demonstrated e.g. in wheat and poplar (Rinaldi et al. 2007, Bhuiyan et al. 2009). Reinforcement and encrustations of the plant cell wall have been described as a general defense reaction at the appressorial contact sites of E. necator in grapevine (Heintz and Blaich 1990). The EPSPS gene induced is located on chromosomes 15 where the resistance loci Ren3 and Ren9 have been identified in QTL analyses. The position of this gene (14.47 to 14.49 Mb on the PN40024 reference genome) is outside of
the confidence internals of the loci Ren3 (9.3 to 9.4 Mb) and Ren9 (1.1 to 3.5 Mb) (Zendler et al. 2017). Genes located in QTL regions in general represent putative receptors of pathogenic signals and need to be active in a basal constitutive expression rather than being significantly induced during pathogen attack.

In this context genes annotated as resistance gene analogs are supposed to act in signal perception and signal transmission during the host/pathogen interaction were investigated (Table). They include an NB-LRR gene as a typical “resistance gene” (Van der Biezen and Jones 1998 a, b). Further a leucine-rich repeat receptor-like serine/threonine-protein kinase (LRR-RLK) gene with a malecin-like carbohydrate-binding domain and a homologue of a calcium-regulated serine/threonine kinase (calcineurin B-like interacting protein kinase gene; CBL-CIPK) were studied.

The NB-LRR transcript appeared to not be significantly induced in either ‘Regent’ or ‘Chardonnay’ at this early point of interaction. This gene is presumed to encode a protein that interacts with effectors released from the attacking pathogen during ETI (Tor et al. 2009). According to its putative function as sensor and trigger of downstream defense pathways, the NB-LRR protein does not need to be present in high amounts but should be constitutively produced at sufficient level in plants to enable them to sense the pathogen effector, as stated above. However, we can not exclude that it may be induced to higher levels at later stages of pathogen ingress, when more effectors of the pathogen entered the host cell.

In contrast, the LRR-RLK gene was moderately induced in ‘Regent’ and to a higher degree in the susceptible grapevines at 10 hpi (Table). LRR-RLKs are considered to be primary sensors of pathogen attack during PTI. They have an extracellular LRR domain linked through a transmembrane signal transmission during the host/pathogen interaction region to an intracellular kinase. Upon binding of a PAMP (e.g. chitin or ergosterol from fungi) heterodimerization with a second “partner” protein is thought to take place. Auto- and trans-phosphorylation reactions transmit the signal to MAP kinase cascades that finally alter transcription in the nucleus (Tor et al. 2009).

The CBL-CIPK may be involved in sensing alterations of intracellular Ca²⁺ concentrations or a specific “Ca²⁺ signature” associated to pathogen attack (Luan 2009). The CBL-CIPK gene appears slightly more induced in ‘Regent’ as compared to the susceptible grapevine (Table). Ca²⁺-influx is one of the early reactions in PTI (Muthamilarasan and Prasad 2013 and references therein). Ca²⁺ can be bound by Calcineurin B-like (CBL-) proteins that specifically interact with CBL-interacting protein kinases. Signaling via Ca²⁺ is involved in several stress conditions and regulatory path-

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**Table**

Results of RT-qPCR. Relative expression (fold induction) and chromosomal localization of 26 genes analyzed by RT-qPCR 10 hpi. Transcripts from the resistant ‘Regent’ and the susceptible ‘Chardonnay’ were compared between non-inoculation and 10 hpi with powdery mildew (*E. necator*).

<table>
<thead>
<tr>
<th>Annotation / abbreviated designation</th>
<th>Regent¹</th>
<th>Std. Error</th>
<th>Chardonnay²</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>myb-related transcription factor, MYB15</td>
<td>11.26</td>
<td>9.324 - 25.379</td>
<td>1.52</td>
<td>0.926 - 2.515</td>
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<td>wrky transcription factor 75, WRKY75</td>
<td>8.57</td>
<td>6.350 - 11.803</td>
<td>1.54</td>
<td>1.396 - 1.700</td>
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<td>probable wrky transcription factor 33-like, WRKY33</td>
<td>7.58</td>
<td>4.976 - 10.793</td>
<td>1.66</td>
<td>1.147 - 2.460</td>
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<tr>
<td>wrky transcription factor 7, WRKY7</td>
<td>3.01</td>
<td>2.592 - 3.463</td>
<td>1.7</td>
<td>1.475 - 2.036</td>
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<tr>
<td>ethylene-responsive element binding factor 2, ERF2</td>
<td>3.63</td>
<td>3.139 - 4.173</td>
<td>1.64</td>
<td>1.529 - 1.796</td>
</tr>
<tr>
<td>ethylene-responsive transcription factor 5, ERF5</td>
<td>2.11</td>
<td>1.816 - 2.449</td>
<td>1.04</td>
<td>0.964 - 1.133</td>
</tr>
<tr>
<td>CZF1, ankyrin repeat; ankyrin repeat-containing domain; Zinc finger CCCH-type, CZF</td>
<td>2.98</td>
<td>2.707 - 3.307</td>
<td>1.17</td>
<td>1.122 - 1.218</td>
</tr>
<tr>
<td>pathogenesis-related protein 10, PR10</td>
<td>49.56</td>
<td>37.796 - 67.789</td>
<td>1.57</td>
<td>1.051 - 2.352</td>
</tr>
<tr>
<td>thaumatin-like protein, PR5</td>
<td>15.56</td>
<td>13.614 - 17.791</td>
<td>2.02</td>
<td>1.770 - 2.313</td>
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<td>beta-1,3-glucanase, PR2</td>
<td>3.90</td>
<td>2.895 - 4.987</td>
<td>6.27</td>
<td>4.131 - 8.792</td>
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<td>hypothetical protein, glycine rich, GRP</td>
<td>2.54</td>
<td>1.921 - 3.284</td>
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<td>1.862 - 4.629</td>
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<td>heat shock protein 70, HSP70</td>
<td>2.48</td>
<td>2.209 - 2.803</td>
<td>6.43</td>
<td>5.182 - 8.067</td>
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<td>probable LRR-receptor-like serine-threonine protein kinase, LRR-RLK</td>
<td>1.69</td>
<td>1.078 - 2.686</td>
<td>4.33</td>
<td>2.832 - 6.521</td>
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<tr>
<td>CBL-interacting protein kinase 11 isoform X1 (<em>V. vinifera</em>), CBL-CIPK</td>
<td>1.66</td>
<td>1.143 - 2.383</td>
<td>1.28</td>
<td>0.867 - 1.804</td>
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<td>Disease resistance protein, NB-ARC-LRR</td>
<td>0.84</td>
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<td>3-deoxy-d-arabino-heptulosonate 7-phosphate synthase, DAHP</td>
<td>10.78</td>
<td>5.642 - 21.164</td>
<td>2.32</td>
<td>1.366 - 3.541</td>
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<tr>
<td>5-enolpyruvylshikimate-3-phosphate synthase, EPSPS</td>
<td>2.96</td>
<td>2.458 - 3.615</td>
<td>1.66</td>
<td>1.591 - 1.738</td>
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<td>phenylalanine ammonia-lyase, PALI</td>
<td>11.77</td>
<td>11.374 - 12.174</td>
<td>0.91</td>
<td>0.739 - 1.128</td>
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<td>cinnamate-4-hydroxylase, C4H</td>
<td>4.20</td>
<td>3.815 - 4.626</td>
<td>1.48</td>
<td>1.322 - 1.680</td>
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<td>probable glutathione S-transferase GSTU25, GST</td>
<td>5.23</td>
<td>4.914 - 5.579</td>
<td>1.50</td>
<td>1.397 - 1.610</td>
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<tr>
<td>molybdoenzyme cofactor</td>
<td>1.79</td>
<td>1.690 - 1.891</td>
<td>0.87</td>
<td>0.792 - 0.962</td>
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<tr>
<td>dihydrofolate reductase, DHFR</td>
<td>5.44</td>
<td>5.305 - 5.588</td>
<td>0.58</td>
<td>0.468 - 0.731</td>
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<td>cysteine histidine-rich C1 domain-containing protein, CHR-C1</td>
<td>2.96</td>
<td>2.859 - 3.067</td>
<td>3.36</td>
<td>3.173 - 3.542</td>
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<tr>
<td>hypothetical protein</td>
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<td>6.087 - 9.113</td>
<td>1.47</td>
<td>1.296 - 1.667</td>
</tr>
<tr>
<td>calnexin homolog CNX1, CNX</td>
<td>4.86</td>
<td>4.378 - 5.415</td>
<td>2.15</td>
<td>1.845 - 2.507</td>
</tr>
</tbody>
</table>

¹ Relative gene expression (fold induction) determined by RT-qPCR of Regent challenged versus non-challenged with powdery mildew.

² Relative gene expression (fold induction) determined by RT-qPCR of Chardonnay challenged versus non-challenged with powdery mildew.
ways including pathogen interactions in plants (Boudsocq et al. 2010, Batistic and Kudla 2012). Furthermore it is linked to the generation of reactive oxygen species (ROS) and participates in the regulation of MAP kinases during plant immunity (Mazars et al. 2010). Ca" signaling may thus well be operating during this early host/pathogen interaction. This presumption is in compliance with the three-fold induction of the gene for transcription factor CZF1/ZFAR1 observed 10 hpi in resistant 'Regent' (Table). In Arabidopsis thaliana it was shown that this factor is phosphorylated by Ca"-dependent protein kinases during herbivore wounding response signaling (Kanchiswamy et al. 2010).

These genes are thought to be involved in pathogen perception and signal transduction. However, they do not fall in the confidence intervals of resistance loci Ren3 or Ren9. Thus they do not present the primary targets of signal molecules from the pathogen attack.

The most important regulatory components differentially induced were the genes encoding transcription factors MYB15, WRKY75, WRKY33, WRKY7, ERF2 and ERF5 (Table).

The MYB15-annotated gene was strongly induced among the transcription factors in resistant 'Regent' (Table). MYB15 belongs to the family of R2R3-MYB transcription factors and has been shown to be involved in the regulation of the stilbene biosynthesis pathway. Stilbenes like resveratrol and its derivatives are important phytoalexins in grapevine. They are known to be induced during various host/pathogen interactions, but also by abiotic stress and developmental processes such as véraison (a specific stage of fruit ripening when berries begin to soften), coincidental with MYB15 (or the closely related MYB14) expression. MYB15 contains a stress-response signal SG2 at its C-terminal end and is predicted to be targeted to the nucleus. Unlike other MYB transcription factors it works independently from co-factors such as WD40 or BHLH (basic helix-loop-helix)-type factors in regard to the induction of stilbene biosynthesis (Holl et al. 2013). In inoculation experiments with the downy mildew pathogen P. viticola, significant induction of MYB15 transcripts was observed at 48 hpi and its maximal expression occurred at 72 hpi. It appeared still low in the early interaction between 8 and 16 hpi (Holl et al. 2013). Here, in the experiment of powdery mildew challenge, the induction of MYB15 was already 11-fold elevated at 10 hpi in resistant 'Regent' (Table) while no stilbene synthase (STS) gene was among the top 75 genes induced at this point. However, it is possible that the MYB15 expression rises further during the progress of infestation and is followed by STS induction later due to binding of MYB15 to activate the promoters of STS genes. MYB15 has also been speculated to be involved in regulation of the phenylpropanoid-pathway. An elevated STS later due to binding of MYB15 to activate the promoters of the stilbene biosynthesis pathway. Stilbenes like resveratrol and its derivatives are important phytoalexins in grapevine. The grapevine genome contains 73 genes predicted to encode proteins with a single AP2/ERF domain for DNA binding originally identified in homeotic genes of floral development (Jofuku et al. 1994). The grapevine genome contains 73 genes predicted to encode proteins with a single AP2/ERF domain as members of the ERF family (Zhuang et al. 2009). ERF transcription factors are known in Arabidopsis to regulate disease resistance pathways and several are responsive to Jasmonic acid (JA) or Ethylene (Et). ERF transcription factors bind to the target sequence AGCCGCC (the "GCC box") in promoters (Gutterson and Reuber 2004). ERF and ERF6 were shown to have redundant functions in JA/ Et mediated defense against Botrytis cinerea in Arabidopsis (Moffat et al. 2012). Overexpression of either one leads to enhanced transcription of PR-genes. In tobacco, NtERF5 transcription is induced by wounding and attack of the bacterial pathogen Pseudomonas syringae; overexpression of NtERF5 enhanced resistance to Tobacco mosaic virus (Fischer and Dörgé-Laser 2004). These data support the hypothesis that the induction of ERFs 2 and 5 observed after N. vitis challenge in 'Regent' plays a role in defense. The earlier observation that Ethephon, a substance releasing ethylene, protects grapevine from N. vitis infection (Belhadi et al. 2008) is in agreement with a presumptive role of ERF transcription factors in defense pathways.

These results are in contrast to the powdery mildew-inoculation response described for the American Vitis aestivalis accession 'Norton'. In this case only three genes were induced in the resistant grapevine. Genes encoding a 3-hydroxy-3-methylglutaryl coenzyme A reductase, a class III peroxidase and an unknown transcript were up-regul-
ed at 8 and 12 hpi. This weak transcriptome response was hypothesized to be due to an elevated level of endogenous salicylic acid (SA) in 'Norton' which causes constitutive expression of defense-related genes (Fung et al. 2008). In the Chinese powdery mildew resistant wild species accession 'Shang24' from V. quinquangularis however, PR5, PR10 and PAL were also identified among differentially induced genes upon E. necator infection at early stages (12 and 24 hpi; Gao et al. 2012). A set of Central Asian V. vinifera accessions exhibit partial resistance to E. necator and were recently investigated by RNA sequencing and RT-qPCR. In this study 13 genes were differentially expressed at 1 dpi (day post inoculation) in the majority of the seven accessions. They included ERF105, a precursor of PR-1 and five putative receptor-like kinase genes (Amrine et al. 2015).

A global transcriptome analysis on downy mildew (P. viticola) infected susceptible V. vinifera 'Pinot noir' in comparison to downy mildew resistant V. riparia 'Gloire de Montpellier' showed comprehensive transcriptional changes both in the compatible and the incompatible interactions at 12 hpi and later. Many genes were induced to higher levels or specifically in V. riparia as compared to V. vinifera. A "wave" of signal transduction was observed at 12 hpi leading to defense responses. Some genes activated under this challenge are similar to genes observed here as powdery mildew-induced genes, e.g. Ca2+ homeostasis-affecting components, PR-10, WRKY transcription factors, ERFs, genes for aromatic amino acid biosynthesis enzymes and receptor-like kinases (Polesani et al. 2010). Both the ascomycete E. nectar and the oomycete P. viticola are obligate biotrophs that parasite their host cells by the formation of haustoria. Some commonalities between the host defense responses against these two different pathogens are therefore anticipated. In both our report and the description of Polesani et al. (2010) the investigation started with the earliest infestation of the host. Resistance due to a hypersensitive response was observed at a post-penetration stage later during the host-pathogen interaction in 'Regent' (Zendler et al. 2017). It is therefore possible that in these "early" post-infection studies PTI was revealed rather than ETI. The latter requires release of effectors from the pathogen into the host cell which may not yet have happened. A set of candidate effectors from P. viticola has recently been presented (Mestre et al. 2016), but evidence for their secretion into the host cell remains to be shown. Besides this circumstance novel knowledge about the genes involved in PTI contributes to our understanding of plant defense and resistance. It enables a selection of genes which should be considered to supplement the breeding for resistant cultivars by combining them with resistance loci from QTL studies in specific crosses. Modern grapevine breeding employs genetic selection by trait-linked markers (marker assisted selection, MAS; Eibach et al. 2007). For the purpose of marker development from candidate genes it will be necessary to reveal the molecular differences between early inducible genes from resistant grapevine genotypes and the less or late reacting alleles from susceptible grapes. Key regulatory elements like MYB15, WRKY or ERF transcription factors identified here in the case of E. nectar infection with resistant 'Regent' should be a target for further investigation.

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