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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 transcripton factor in regulating the early defense when the fungus starts the interaction with its host by the formation of haustoria.

K e y w o r d s : *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 amounts 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 biotrophic 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 *Muscadinia 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 (Feechan *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

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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 Dangl 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 infestation progress was monitored by microscopy during the early phases after experimental inoculation. RNA prepared from the time 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', 'Chardonnay') were grown *in vitro* under controlled conditions (16 h photoperiod (~ 42 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 24 °C) on 0.5x MS medium (pH 6.2) (Murashige and Skoog 1962, Duchefa M0233, Haarlem, The Netherlands).

For microscopy of spore germination at early stages the three upper leaves from these *in vitro* grown plantlets were transferred adaxial side up onto 0.8 % agar (Merck, Darmstadt, Germany) in water in Petri dishes (Greiner, 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.

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.

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. Non-inoculated plants from five glass vessels of both cultivars were kept under identical conditions as negative control. At 10 hpi leaves were collected, immediately frozen in liquid nitrogen and stored at -70 °C until RNA preparation. Inoculated leaves from susceptible plants were observed during the following hours under binocular lens and a microscope (Zeiss Axiolab, Jena, Germany) to monitor the development of the fungus. Two completely independent biological replications were performed.

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 H_2O). 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 $\alpha = 5$ %.

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 $g \cdot L^{-1}$ Spermidine (Serva) and 2 % ß-mercaptoethanol (Sigma-Aldrich,

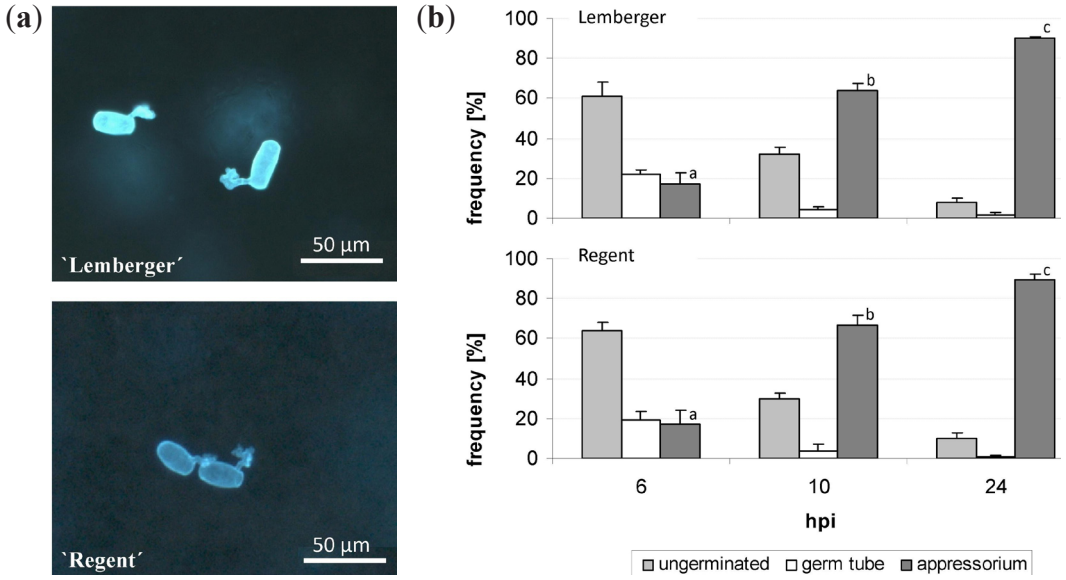

Figure: Early infection stages in susceptible 'Lemberger' and resistant 'Regent'. (**a**): Epifluorescence micrographs of *Erysiphe necator* 10 hpi obtained after staining with "Direct Yellow 96". On *in vitro* leaves of both cultivars (a, 'Lemberger'; b, 'Regent'), germinating conidia formed lobed and mature appressoria indicating a successful onset of a plant-pathogen interaction. Bar equals 50 µm.
(**b**): The development of conidia was followed on detached leaves of resistant 'Regent' and susceptible 'Lemberger' *in vitro* plants inoculated with *E. necator* conidia. Three leaves were observed at 6, 10 and 24 hpi. 100 conidia per leaf were evaluated for the percentage of formation of germ tubes or appressoria. No significant differences between the resistant and the susceptible host could be demonstrated at this early stage.

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 pellet was washed twice with 80 % Ethanol and once with absolute Ethanol (Applichem, Darmstadt, Germany), dried at room temperature and resuspended in 30 mL of RNase-free water. 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 suppliers 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/tgi/cgi-bin/tgi/gimain.pl?gudb=grape and meanwhile shifted to ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/*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 T_m: 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 1mg 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 mL, containing 12.5 mL 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 (C_t) 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 C_t 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 C_t 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). ß-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 check for activation of pathogen-restricting functions. From the 26 genes studied, 22 showed at least a two-fold transcriptional induction in 'Regent' challenged with powdery mildew as compared to the non-inoculated control. Three of the remaining genes showed an induction ratio greater than 1.6. The differential expression levels of these genes are summarized in Table. The majority of the transcripts investigated showed a stronger accumulation in the resistant *Ren3/9* carrier 'Regent' in response to *E. necator* inoculation than in the susceptible cultivar. Exceptions are five genes that were induced to a similar or higher fold-increase in the susceptible cultivar (ß-1,3 glucanase, *GRP*, *HSP-70*, *LRR-RLK* and a *C1* domain-containing protein gene, cf. Table).

The highest induction ratio in 'Regent' at 10 hpi was found for a gene encoding PR-10 on chromosome 5. This gene was induced almost 50-fold in 'Regent' while it was activated only 1.5-fold in the susceptible grapevine. PR-10 is an intracellular cytosolic protein. Several copies are encoded in a gene family as characterized in *V. vinifera* (14 genes in one cluster on chromosome 5 (Lebel *et al.* 2010)) and the Chinese grape species *Vitis pseudoreticulata*. Some variants are known to have DNase and RNase as well as antifungal activity (Wang *et al.* 2014, Xu *et al.* 2013). PR-10 is involved in plant defense and has even been described as being translocated into *Plasmopara viticola* haustoria (He *et al.* 2013), an important oomycete pathogen of grapevine. It thus may play an important role in the defense reaction against *E. necator*.

The second most induced transcript also codes for a pathogenesis related protein, PR-5 (Thaumatin-like protein). It was induced 15.5-fold in 'Regent' but only 2-fold in 'Chardonnay'. The transcript corresponds to one gene of a family of four different thaumatin-like genes annotated on chromosome 2 and spread over a genomic region of 18.9 kb. Thaumatin-like proteins have been found to possess antifungal activity and are known to be involved in plant defense, although their mechanism of action is yet unresolved (Anzlovar and Dermastia 2003, Petre *et al.* 2011, Liu *et al.* 2012). The effect on fungal membrane permeability has been discussed (Anzlovar and Dermastia 2003).

The third group of induced genes codes for key enzymes of the shikimate and phenylpropanoid pathways including DAHP (3-deoxy-d-arabino-heptulosonate 7-phospate synthase), EPSPS (5-enolpyruvylshikimic acid 3-phosphate synthase), PAL (phenylalanine-ammonium lyase) and COMT (caffeic acid o-methyltransferase). DAHP mediates the condensation of phosphoenol pyruvate and erythrose 4-phosphate to yield shikimate, which is the precursor of aromatic amino acids (tryptophan, phenylalanine and tyrosine) that are formed via EPSPS. Phenylalanine can be processed further by PAL yielding *trans*-cinnamic acid, the entry point of the core phenylpropanoid pathway. Downstream branches of this metabolic pathway yield major flavonoid groups and related compounds such as stilbenes, the phytoalexins of grapevine, or caffeic acid and other simple phenylpropanoids including salicylic acid (SA). It is well known that DAHP, EPSPS and PAL are transcriptionally activated by wounding and pathogen attack. COMT can act on caffeic acid to produce monolignols and precursors of lignin biosynthesis (Bowsher *et al.* 2008). These may participate in cell wall reinforcement at the infection sites and help to contain 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 chromosome 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

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*)

Annotation / abbreviated designation	Regent[1]	Std. Error	Chardonnay[2]	Std. Error
myb-related transcription factor, MYB15	11.26	9.324 - 25.379	1.52	0.926 - 2.515
wrky transcription factor 75, WRKY75	8.57	6.350 - 11.803	1.54	1.396 - 1.700
probable wrky transcription factor 33-like, WRKY33	7.58	4.976 - 10.793	1.66	1.147 - 2.460
wrky transcription factor 7, WRKY7	3.01	2.592 - 3.463	1.7	1.475 - 2.036
ethylene responsive element binding factor 2, ERF2	3.63	3.139 - 4.173	1.64	1.529 - 1.796
ethylene-responsive transcription factor 5, ERF5	2.11	1.816 - 2.449	1.04	0.964 - 1.133
CZF1, ankyrin repeat; ankyrin repeat-containing domain; Zinc finger CCCH-type, CZF	2.98	2.707 - 3.307	1.17	1.122 - 1.218
pathogenesis-related protein 10, PR10	49.56	37.796 - 67.789	1.57	1.051 - 2.352
thaumatin-like protein, PR5	15.56	13.614 - 17.791	2.02	1.770 - 2.313
beta-1,3-glucanase, PR2	3.90	2.895 - 4.987	6.27	4.131 - 8.792
hypothetical protein, glycine rich, GRP	2.54	1.921 - 3.284	2.82	1.862 - 4.629
heat shock protein 70, HSP70	2.48	2.209 - 2.803	6.43	5.182 - 8.067
probable LRR-receptor-like serine-threonine protein kinase, LRR-RLK	1.69	1.078 - 2.686	4.33	2.832 - 6.521
CBL-interacting protein kinase 11 isoform X1 (*V.vinifera*), CBL-CIPK	1.66	1.143 - 2.383	1.28	0.867 - 1.804
Disease resistance protein, NB-ARC-LRR	0.84	0.700 - 1.037	1.27	1.059 - 1.508
3-deoxy-d-arabino-heptulosonate 7-phosphate synthase, DAHP	10.78	5.642 - 21.164	2.32	1.366 - 3.541
5-enolpyruvylshikimate-3-phosphate synthase, EPSPS	2.96	2.458 - 3.615	1.66	1.591 - 1.738
caffeic acid o-methyltransferase, COMT	12.13	8.492 - 18.590	1.99	1.406 - 2.719
phenylalanine ammonia-lyase, PAL1	11.77	11.374 - 12.174	0.91	0.739 - 1.128
cinnamate-4-hydroxylase, C4H	4.20	3.815 - 4.626	1.48	1.322 - 1.680
probable glutathione S-transferase GSTU25, GST	5.23	4.914 - 5.579	1.50	1.397 - 1.610
molybdopterin cofactor	1.79	1.690 - 1.891	0.87	0.792 - 0.962
dihydrofolate reductase, DHFR	5.44	5.305 - 5.588	0.58	0.468 - 0.731
cysteine histidine-rich C1 domain-containing protein, CHR-C1	2.96	2.859 - 3.067	3.36	3.173 - 3.542
hypothetical protein	7.44	6.087 - 9.113	1.47	1.296 - 1.667
calnexin homolog CNX1, CNX	4.86	4.378 - 5.415	2.15	1.845 - 2.507

[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.

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 that 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 malectin-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 (Tör *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 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 (Tör *et al.* 2009).

The CBL-CIPK may be involved in sensing alterations of intracellular Ca^{2+} concentrations or a specific "Ca^{2+} signature" associated to pathogen attack (Luan 2009). The *CBL-CIPK* gene appears slightly more induced in 'Regent' as compared to the susceptible grapevine (Table). Ca^{2+}-influx is one of the early reactions in PTI (Muthamilarasan and Prasad 2013 and references therein). Ca^{2+} can be bound by Calcineurin B-like (CBL-) proteins that specifically interact with CBL-interacting protein kinases. Signaling via Ca^{2+} is involved in several stress conditions and regulatory path-

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^{2+} 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^{2+}-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 (Höll *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 (Höll *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 expression of PAL was observed in MYB15-expressing transgenic hairy roots (Höll *et al.* 2013). This finding is in agreement with our data showing 11.7-fold induction of PAL concurrent with the 11-fold induction of MYB15 at 10 hpi.

WRKY75, WRKY33 and WRKY7 genes were clearly induced in resistant 'Regent' but only moderately in susceptible 'Chardonnay' (Table). WRKY transcription factors are characterized by their "WRKY"-DNA binding domain and a special zinc-finger structure at the C-terminus. They bind to the W-box sequence (TTGACC/T) in the promoters of responsive genes (Rushton *et al.* 2010). More than 60 WRKY genes have been annotated in the model genome sequence of grapevine PN40024 (http://genomes.cribi.unipd.it). They are implicated in various regulatory pathways in plants including responses to pathogens and plant immunity. Both PTI and ETI involve WRKY factors (Rushton *et al.* 2010). A recent bioinformatic analysis followed by RT-qPCR investigated 59 WRKY genes in *Vitis vinifera* and found two of them significantly induced upon *E. necator* inoculation. Several WRKY transcripton factor genes were involved both in abiotic and biotic stresses (Wang *et al.* 2014). WRKY33 from 'Regent' has been recently characterized in the context of downy mildew resistance. It was identified as a class I WRKY TF and promoted the expression of *PR10*. Its ectopic expression in grapevine leaves resulted in a significant reduction of *P. viticola* sporulation (Merz *et al.* 2015). Two WRKY transcription factor genes were described to be involved in *E.necator* resistance of the Chinese wild grapevine species *Vitis pseudoreticulata* (Li *et al*. 2010). A comprehensive study in *Arabidopsis* revealed a regulatory network of interaction and trans-phosphorylation of several WRKY factors, Ethylene-responsive transcription factors (ERF) and chitin-responsive MAP kinases. This network included WRKY33, WRKY72 and ERF5 (Son *et al.* 2012). In conformance with this data the ethylene-responsive transcription factors ERF2 and ERF5 were up-regulated predominantly in 'Regent' challenged with *E. necator* at 10 hpi. Induction ratios reached about twice the level of susceptible 'Chardonnay' (Table). ERF transcription factors 2 and 5 have an AP2 (*APETALA2*) 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). ERF5 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 Dröge-Laser 2004). These data support the hypothesis that the induction of ERFs 2 and 5 observed after *E. necator* challenge in 'Regent' plays a role in defense. The earlier observation that Ethephon, a substance releasing ethylene, protects grapevine from *E. necator* infection (Belhadj *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-regulat-

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. Ca^{2+} 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. necator* and the oomycete *P. viticola* are obligate biotrophs that parasitize 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. necator* infection with resistant 'Regent' should be a target for further investigation.

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