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Research article

Transcriptomic changes following the compatible interaction *Vitis vinifera*–*Erysiphe necator*. Paving the way towards an enantioselective role in plant defence modulation

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ABSTRACT

The compatible interaction between *Erysiphe necator* and *Vitis vinifera* induces significant alterations in the host transcriptome, affecting essentially those genes involved in signalling and secondary metabolite biosynthetic pathways. The precise transcriptomic changes vary from the early events to later stages of infection. In the present work, suppressive subtraction hybridization (SSH) was used to identify several differentially expressed transcripts in symptomatic and asymptomatic leaves from powdery mildew infected grapevines following a long term interaction. The detected transcripts show little or no correlation with similar expression studies concerning the early stages of infection which suggests distinct host responses occur before and after the infection is established. The transcription level of thirteen genes was assessed through qRT-PCR using appropriately selected and validated normalization genes. With one exception, all these genes underwent moderate levels of differential transcription, with $\log_2$-fold change values ranging from −2.65 to 4.36. The exception, a dirigent-like (*DIR*) protein, was upregulated over 180 fold in symptomatic leaves, suggesting an important role for stereochemical selectivity in the compatible interaction *E. necator*–*V. vinifera*. *DIR* copy number was determined in the genome of three grapevine cultivars exhibiting high (Carignan), moderate (Fernão Pires) and low (Touriga Nacional) sensitivity to *E. necator*. It was found to be a two-copy gene in all cultivars analyzed. Further analysis involving DIR metabolic neighbourhood transcripts was performed. The possible physiological significance of the detected *DIR* upregulation is discussed.

1. Introduction

Erysiphe necator, the causal agent of powdery mildew (PM) in *Vitis vinifera*, represents, from an economic point of view, one of the most devastating diseases affecting grapevine worldwide [1]. Although its propagation can be minimized by chemically-synthesized antifungal compounds, the inevitable increase in fungicides used to prevent and contain this disease comprises a potential environmental threat [2]. It is, therefore, of utmost importance to improve our knowledge on the molecular and biochemical mechanisms underlying *V. vinifera*–*E. necator* interaction. Numerous studies regarding this and other host–pathogen systems have shown that complex transcriptomic and subsequent metabolic changes occur within the host in response to the pathogen [3]. As soon as the plant detects the presence of a pathogen, the induced defence response is triggered. Cell surface-located receptors are responsible for initial pathogen recognition, detecting pathogen-associated molecular patterns (PAMPs) and activating a kinase cascade-mediated signal transduction [4]. The resulting modifications range from upregulation of constitutive defence-related genes to transcriptionally induced or post-transcriptionally regulated disease-associated proteins, which may lead to PAMP-triggered immunity (PTI) [5]. Even though PTI can, occasionally, protect the plant from microbial invasion and/or proliferation during compatible interactions, it constitutes a nonhost-resistance mechanism against nonadapted pathogens [6].

When dealing with compatible plant–pathogen interactions, PTI is often, at least partially disabled through the use of effector proteins [5,7]. This is particularly relevant in pathosystems where the pathogen is an obligate biotrophic fungus, as in *V. vinifera*–*E. necator* interaction. Unlike facultative biotrophic or hemibiotrophic fungi which can survive outside the host or switch to necrotrophy,

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E. necator and other obligate biotrophic pathogens are entirely dependent on living plant tissue for their growth and propagation [8]. Therefore, they develop a highly sophisticated interaction with their hosts. Apparently, these pathogens have the ability to suppress or durably avoid pre-formed and induced host defences including hypersensitive response. In addition, they seem to be able to redirect the host metabolism according to their nutritional needs [9,10].

Among all *Vitis* species, *V. vinifera* cultivars display the highest susceptibility to powdery mildew. Other species such as *Vitis labrusca*, *Vitis rupestris* and *Vitis aestivalis* exhibit several degrees of powdery-mildew resistance which can be somehow related to their co-evolution with *E. necator* during a long course of time [11]. A comparative transcriptomic study between *V. vinifera* and *V. aestivalis* suggests that the differential disease susceptibility among *Vitis* species goes beyond genome variation and is more likely to be determined by transcriptional regulation [12]. Recent evidence supporting this hypothesis shows a significant transcriptomic difference between compatible and incompatible interactions involving grapevine and *E. necator*. The differential gene expression induced by *E. necator* was observed to be limited to three transcripts in *V. aestivalis* against 625 PM-responsive genes found in *V. vinifera* up to 48 h postinoculation (hpi). The reason why such a weak PM-induced response occurs in *V. aestivalis* may be connected to its constitutive transcriptomic profile which is already defence-oriented when compared to *V. vinifera* [13]. The observed transcriptome changes in *V. vinifera* during the course of infection is consistent with the theory that these sophisticated pathogens are, somehow, able to circumvent host defence/recognition mechanisms [9]. Many differentially expressed defence-related proteins reached their maximum levels at 12 hpi and then declined as the fungal infection became established [13]. Similar results were reported for barley (*Hordeum vulgare*) powdery mildew, where compatible and incompatible interactions with the pathogen *Blumeria graminis* caused analogous plant expression patterns during the first 16 h of infection. After this period, the expression of some of these defence-related genes declined to lower levels in susceptible plants, at 24 and 32 hpi [14]. Many of these genes are involved in plant secondary metabolism which, in *V. vinifera*–*E. necator* interaction, apparently shift the metabolism towards phenylpropanoid synthesis via the pentose phosphate and shikimate pathways. As a result, lignin, stilbene and dihydroflavonol metabolic branches are also upregulated.

Among all the differentially expressed reported genes, dirigent proteins (DIR) represent a particularly interesting defence-related multigene family [13]. DIR proteins (from Latin: *dirigere* = to guide or align) are proteins devoid of catalytic activity which dictate the stereochemistry of reactions catalyzed by other proteins. They were first reported as auxiliary proteins in lignan biosynthesis where they were shown to regio-stereochemically control the monolignol radical coupling catalyzed by peroxidases, namely dimerization of coniferyl alcohol to afford (+)-pinoresinol [15]. DIR discovery and their subsequent detection in all land plants examined to date explain the usually observed lignan optical activity in biological samples [16]. Lignans are characterized for their recognized antifungal activity, but also their chemical structure, with several chiral centres, allowing regio-stereochemical diversity along their biosynthetic route [17]. The observation that, under pathological conditions, this diversity is restricted to favour the formation of a single enantiomer highlights the potential importance of DIR enantioselective character in plant defence. Previous studies on lignan properties have already shown differential antimicrobial activity between enantiomers, one being up to four times more toxic than the other [18]. So far, a reasonable number of DIR/DIR-like genes have been identified and phylogenetically grouped into six distinct subfamilies. Yet, little is known about the type or number of reactions they might assist. Until recently, DIR biochemical/physiological functions had only been assigned to members of the DIR-a subfamily, involving the guidance of coniferyl alcohol dimerization to afford (+)-pinoresinol [19]. Lately, members of DIR-b/d subfamily in cotton were also characterized as participating in terpenoid synthesis, namely in the enantioselective dimerization of hemigossypol to form (+)-gossypol [20]. There is also some evidence that DIR might be related with lignin biosynthesis, though this still remains a controversial issue [21]. Apart from those, and given the several thousand known plant lignans, DIR may participate in lignan formation other than (+)-pinoresinol.

In this study, we report the detection of several *V. vinifera* defence-related genes whose expression was found to be affected following the long term interaction with *E. necator*. The comparison between our results and similar studies performed during this plant–pathogen interaction indicates that distinct host modifications occur during the initial and late stages of infection. The differential gene expression between symptomatic and asymptomatic leaves of powdery mildew infected grapevines was confirmed and quantified by qRT-PCR using previously validated normalization genes. Among the detected transcripts a DIR-like gene was identified whose differential expression highlights its potential importance in this plant–pathogen interaction. We further investigated whether *V. vinifera DIR* gene dosage could be related to *E. necator* susceptibility, determining genomic *DIR* copy number for grapevine cultivars with different PM susceptibility degrees. *DIR* was found as a two-copy gene in all three cultivars analyzed. Moreover, an attempt was made to correlate the observed *DIR* upregulation with the relative transcript levels of several genes participating in the metabolic surroundings of coniferyl alcohol, the putative DIR "substrate". Our data suggest that either an alternative lignan biosynthetic pathway exists or DIR may be involved in reactions other than the dimerization of coniferyl alcohol.

2. Results

2.1. SSH cDNA libraries

To identify differentially expressed genes between symptomatic (S) and asymptomatic leaves (A) from powdery-mildew infected grapevines, two cDNA libraries were constructed from forward and reverse suppressive subtraction hybridization (SSH) using S and A samples as testers, respectively. A total of 273 clones from both forward and reverse subtractions were screened for relative transcript abundance in the subtracted cDNA pools. Significant dot-blot hybridization differences were observed for 62 clones for which the corresponding cDNA inserts were sequenced. Sequence analysis revealed 28 non-redundant grapevine transcripts (Table 1) and three *E. necator* transcripts. Most inserts ranged from 300 to 800 bps. High-quality cDNA sequences obtained for 24 transcripts were deposited and made accessible in the dbEST NCBI database. tBLASTx analysis allowed us to assign a predicted function to 19 transcripts due to their very high similarity with known function genes from other plant species. Three of the selected transcripts revealed homologies with grapevine predicted proteins of unknown function. Six sequences showed no significant similarity to known or putative function genes but were homologues to grapevine genomic sequences. The presence of poly(A) tails in the latter confirms they represent grapevine transcripts and thus, new uncharacterized genes.

2.2. Validating internal controls for qRT-PCR

To perform the qRT-PCR gene expression studies between S and A leaf samples, the identification of the most suitable housekeeping genes to be used as internal controls was a first priority. The

Table 1
Differentially expressed transcripts detected by SSH of symptomatic (S) and asymptomatic leaves (A) from powdery-mildew infected grapevines.

Clone ID (accession number)	Subtraction library[a]	Predicted function	tBLASTx	*E* value
F1B5 (KC748395)	Forward	Glycerol-3-phosphate dehydrogenase-like	XM_002284443	9E−141
F1C5 (KC748400)	Forward	Beta-amyrin synthase/triterpene synthase	XM_002269309	1E−24
F1A11 (KC748406)	Forward	Resveratrol-*O*-methyltransferase	XM_002281445	8E−39
F1D5 (KC748408)	Forward	Mitogen-activated protein kinase	XM_003633911	1E−149
F1E6 (KC748418)	Forward	Dirigent-like protein	XM_002276412	2E−80
F1A12 (KC748407)	Forward	Calcium-binding EF hand family protein	AM476824	2E−142
F2C4 (KC748411)	Forward	Tyrosine kinase	XM_003635112	2E−70
F2C6 (KC748412)	Forward	Wall-associated kinase	XM_003631669	4E−82
F1C7 (KC748413)	Forward	Potassium transporter	XM_002264915	1E−75
F1A3 (KC748403)	Forward	Ribosomal protein s15a	XM_003632560	4E−95
F1D11 (KC748415)	Forward	*Vitis vinifera* − unknown/uncharacterized	XM_002277671	4E−94
F2A4	Forward	*Vitis vinifera* − unknown/uncharacterized	XM_003632258	6E−98
F2A5 (KC748404)	Forward	*Vitis vinifera* − unknown/uncharacterized	XM_003631832	8E−112
F1A2	Forward	*Vitis vinifera* contig VV78X174488.4	AM441276	1E−87
F1C2 (KC748409)	Forward	*Vitis vinifera* contig VV78X257382.43	AM476899	6E−136
F1C11	Forward	*Vitis vinifera* contig VV78X054516.4	AM482143	3E−36
F2D1 (KC748414)	Forward	*Vitis vinifera* contig VV78X057541.5	AM441985	2E−102
F1E5 (KC748417)	Forward	*Vitis vinifera* contig VV78X104455.3	AM484817	6E−112
R1D10 (KC748416)	Reverse	Oligopeptide transporter protein	XM_002274130	7E−130
R2C3 (KC748410)	Reverse	Hormone-sensitive lipase/gibberellic acid receptor	XM_002265728	8E−59
R1C7 (KC748397)	Reverse	Cinnamoyl-CoA reductase, putative	XM_002272413	1E−138
R1D7 (KC748401)	Reverse	Zinc-finger RING-type protein-like	XM_002285229	1E−154
R1C11 (KC748402)	Reverse	Oligouridylate binding protein	XM_002276972	3E−151
R2A10 (KC748405)	Reverse	Carbohydrate transporter	XM_002278696	1E−62
R1A8 (KC748398)	Reverse	GPI-anchored protein	XM_002272370	8E−119
R1C2	Reverse	MYB/MYC transcription factor	XM_002282950	9E−120
R1B11 (KC748399)	Reverse	ATPase alpha subunit	GQ220323	9E−155
R1C1 (KC748396)	Reverse	*Vitis vinifera* contig VV78X123770.2	AM425900	4E−126

[a] Forward and reverse SSH were performed using S and A samples as testers, respectively.

candidate genes were evaluated according to their expression stability under the particular experimental conditions tested in the present study. Six commonly used housekeeping genes [22–24] were selected and their relative transcript abundance in S and A cDNA samples was subsequently evaluated using geNorm software [25]. The expression stability for ribosomal protein L2 (*L2*), elongation factor 1 alpha (*EF1alpha*), cyclophilin (*Cyp*), phosphoenolpyruvate carboxylase (*PEPcase*), ubiquitin conjugating enzyme (*UBC*) and vacuolar ATP synthase subunit G (*VAG1*) was tested. For each candidate gene, a gene-stability parameter (*M*) was determined (Fig. 1A) which reflects its expression variation between samples by pairwise comparison with all genes under analysis. Genes with the lowest *M* values are the most stably expressed. This analysis revealed *UBC* and *VAG1* as the best ranked housekeeping control genes for this study, immediately followed by *PEPcase* (Fig. 1A).

To determine the optimal number of internal control genes required for reliable normalization, geNorm was used to calculate the pairwise variation ($V_{n/n+1}$) between two sequential normalization factors (NF_n and NF_{n+1}) (Fig. 1B). $V_{n/n+1}$ reflects the accuracy changes accompanying the inclusion of an $(n + 1)$th gene as internal control. The lower the *V* value, the more stable is the corresponding normalization factor. A cut-off threshold was set at $V = 0.15$ below which the additional control gene has no significant effect in data normalization [25]. As shown in Fig. 1B, under our experimental conditions, $V_{2/3}$ was 0.13, which was below the cut-off threshold. Thus, the addition of a third control gene has negligible effects on the normalization factor and, therefore, the use of the two most stable genes as internal controls, i.e. *UBC* and *VAG1*, is enough for reliable data normalization.

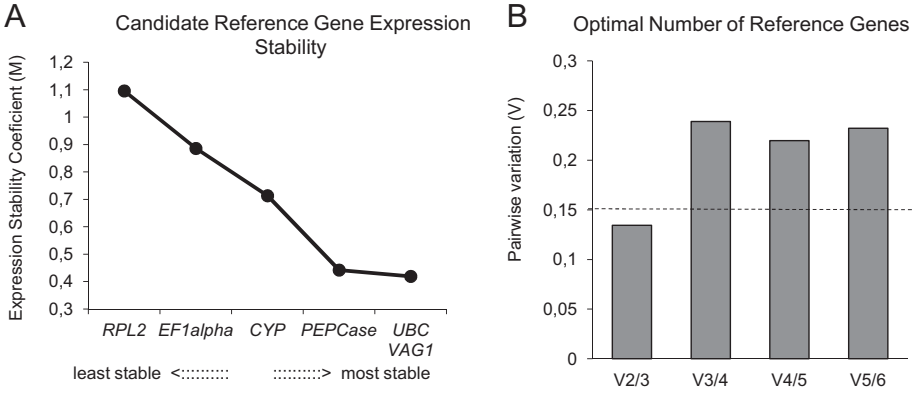

Fig. 1. Evaluation of normalization genes for qRT-PCR. Data were obtained from PCR triplicates of four biological replicates. (A) geNORM ranking of the six evaluated reference genes according to their average expression stability values, *M*, in symptomatic and asymptomatic leaves from powdery mildew infected grapevines. Genes with the lowest *M* values are the most stably expressed throughout the tested conditions. (B) Determination of the optimal number of control genes for normalization using pairwise variation analysis ($V_{n/n+1}$) between the normalization factors NF_n and NF_{n+1}. A cut-off threshold of 0.15 indicates that the addition of a third control gene has no significant effect in data normalization.

2.3. Differential gene expression quantification by real-time RT-PCR

To confirm and quantify the differential gene expression levels detected by SSH, relative transcript quantification between S and A samples was performed using qRT-PCR. PCR primers were designed for all 19 transcripts (Table 2) for which a predicted function could be established. Amplification specificity for each primer pair was evaluated by both BLASTn and melting curve analysis. Yet, eight genes were excluded from the study either due to unspecific amplifications or primer-dimer formation. In addition to the selected genes, the relative expression levels of actin and ribosomal protein L2, two widely used internal controls, were also measured to evaluate their adequacy as normalization factors in qRT-PCR studies on this host–pathogen interaction. The relative gene expression levels between S and A mRNA samples is represented in Fig. 2.

Table 2
qRT-PCR primers.

Primer		Sequence 5′ to 3′	E^a
Triterpene synthase	FW	TCACTCCATCAACCTCAGAAG	nd
	RV	CCCAGAAGAGCGAGAAGAG	
Resveratrol-*vO* -methyltransferase	FW	TGAGGTTCAAGGTTGGAATATG	nd
	RV	TGGTTGTGGATGATGTCTGG	
DIR	FW	CCAGCACTCATACAACTTAATATCAAC	nd
	RV	CTCAACAGCACAGACTTTCAGG	
Glycerol-3-phosphate dehydrogenase	FW	GCAACACGCTCAAG	nd
	RV	TGGCTGACACCTATG	
MAPK	FW	CAACTATGACACAAAGGAAGAAG	nd
	RV	CATTTACTGAAGAGAATATCAAAGAAC	
Oligopeptide transporter	FW	TATTGGAATAATCTACAGCGTGATAGTG	nd
	RV	GAAGACGAAGGCGAGCAGAG	
Gibberellic acid receptor	FW	AGTAGAAGCCGATTGTTGC	nd
	RV	AGAGTCTTGTTGTGGTTGC	
Inositol transporter	FW	GTAATAAGGTATCCAGTTGTTCAATG	nd
	RV	CTTCCTCTCAAATTCAGTCTATCG	
Cinnamoyl-CoA reductase	FW	GGTTGGAGATGGTGAGATG	nd
	RV	CTTGACGAGAGAAACTGGAG	
Oligouridylate binding protein	FW	AAGGTGCTGGAGGTAACG	nd
	RV	ATGTCGGTGGAGGTCAAC	
Zinc-finger RING-type protein	FW	TGAGATAGCCAGACAAGTAG	nd
	RV	TTGCCTCCACCATTGC	
Actin (XM_002282480.2)	FW	TGGATTCTGGTGATGGTGTGAGTC	nd
	RV	CAATTTCCCGTTCAGCAGTAGTGG	
L2 (AJ441290.2)	FW	TCTACTTCAACCGATATGC	0.89
	RV	CCACCTGTCCGACTG	
PEPCase (AF236126.1)	FW	CCTCCTCCTCCAGATTGC	0.86
	RV	GGCTTGCTTGATTCCATTATC	
UBC (EE253706)	FW	CATAAGGGCTATCAGGAGGAC	0.95
	RV	TGGCGGTCGGAGTTAGG	
Cyp (ES880796)	FW	ACAGCCAAGACCTCGTG	0.90
	RV	GCCTTCACTGACCACAAC	
VAG1 (XM_002281110.1)	FW	TTGCCTGTGTCTCTTGTTC	1.11
	RV	TCAATGCTGCCAGAAGTG	
EF1α (GU585871.1)	FW	GAACTGGGTGCTTGATAGGC	0.87
	RV	AACCAAAATATCCGGAGTAAAAGA	
UFGT (JF522535.1)	FW	GGTGGTTTTACCTGCTAATTTGTT	nd
	RV	GTGAGAAGAGCGAGTTTAGGTTTC	
DFR (AY780886.1)	FW	TCATCACTATCATACCGACTCTTGT	nd
	RV	CCTGCCGTATAATTGAATGAGC	
AR (XM_002282806.1)	FW	TCTATCAGCACCATCACAGCCAAGG	nd
	RV	CCAACCACCCACTCTCGTCTTCC	
ConGlu (XM_002264642.2)	FW	TCTCTTGGAGTCAACAGCTACCG	nd
	RV	TCACTTCTCCAAATCTCCCTTCGG	
PRR[b]	FW	TTCTTGTDGTGGGTGGVACAGGSTA	nd
	RV	ACCAYATYTACTTTCTTCACWGCTT	
CaOMT[b]	FW	TCSMCCRGTATGAYCCCARMAACTA	nd
	RV	TGGKGAYCTKAGCAGCAATCT	
CAD[b]	FW	AAATGSGTAGCCTTGRGACTGA	nd
	RV	TCYGATCCCACCTCCACC	
UDP-GT (XM_002273320.1)	FW	ACTCCTCATCTCCCTAACTTGCTG	nd
	RV	TTCATGGCCGAGATTGCAGAG	
F5H (XM_002272608.2)	FW	TCCGACTCCACCCACCTATT	nd
	RV	GCCCGCTTTGAGAAACCTTG	

nd – Not determined.
[a] Primer pair amplification efficiency.
[b] Degenerate primer.

With the exception of KC748416, representing an oligopeptide transporter, all SSH-detected transcripts were found to be present in significantly different amounts in symptomatic and asymptomatic samples. The agreement observed in all cases between the cDNA library, from where the transcripts were identified (forward or reverse SSH), and their correspondent up or downregulation also validates the subtraction process (Table 1, Fig. 2). Overall, the analyzed genes present slightly moderate levels of differential expression with $\log_2$(fold change) values ranging from −2.65 to 4.36 for L2 ribosomal protein and glycerol-3-phosphate dehydrogenase, respectively. Among all the transcripts, the dirigent-like protein (*DIR*) (KC748418) stands out of the remaining due to the magnitude of overexpression in symptomatic leaves when compared to asymptomatic ones. Its upregulation reaches over 180 fold change, representing a variation 10–150 times larger than that observed for the remaining transcripts.

2.4. Genomic DIR copy number determination in V. vinifera cultivars

To assess whether *DIR* gene dosage could be somehow relevant in *V. vinifera–E. necator* interaction, qPCR was used to determine the *DIR* copy number in three *V. vinifera* cultivars with different powdery mildew susceptibilities. Genomic DNA from the cultivars Carignan, Fernão Pires and Touriga Nacional was used as template for *V. vinifera* cultivars with high, moderate and low powdery mildew susceptibility, respectively. The powdery mildew susceptibility degree of each cultivar was assessed according to the empirical ranking provided by Dr. Antero Martins and co-workers (ISA/PORVID), which is based on their wide viticulture experience and field observations.

Given the individual DNA quality patterns obtained from the three *V. vinifera* cultivars, accurate spectrophotometric DNA quantification was not feasible and thus absolute quantification of *DIR* gene copy number was not carried out. Instead, a relative quantification procedure was selected using cultivar Pinot Noir genomic DNA as calibrator and three single-copy genes as reference. According to the grapevine genome sequence available at Genoscope (http://www.genoscope.cns.fr/spip/), *DIR* is a two-copy gene in Pinot Noir, while UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*UFGT*), dihydroflavonol reductase (*DFR*) and anthocyanidin reductase (*AR*) are products of single-copy genes. *DIR* copy number was calculated (Fig. 3) based on the $E^{-\Delta Ct}$ ratios between *DIR* and single-copy genes according to the following equation:

$$\text{DIR copy number} = \frac{E_{DIR}^{\Delta Ct(\text{calibrator}-\text{cultivar})}}{E_{ref}^{\Delta Ct(\text{calibrator}-\text{cultivar})}} \times 2$$

where E = amplification efficiency of the gene (*DIR* or *reference*) and ΔCt = calibrator Ct − cultivar Ct from the respective gene.

No significant differences in the determined $E^{-\Delta Ct}$ ratios were observed for each of the three cultivars (Fig. 3), indicating that *DIR* is a two-copy gene in the genomes of the cultivars Carignan, Fernão Pires and Touriga Nacional. Therefore, the different degree of susceptibility exhibited by *V. vinifera* cultivars to *E. necator* infection does not seem to be related to DIR copy number.

2.5. Coniferyl alcohol branching genes

Up to date, almost all the described DIR-assisted reactions involve coniferyl alcohol dimerization to afford (+)-pinoresinol

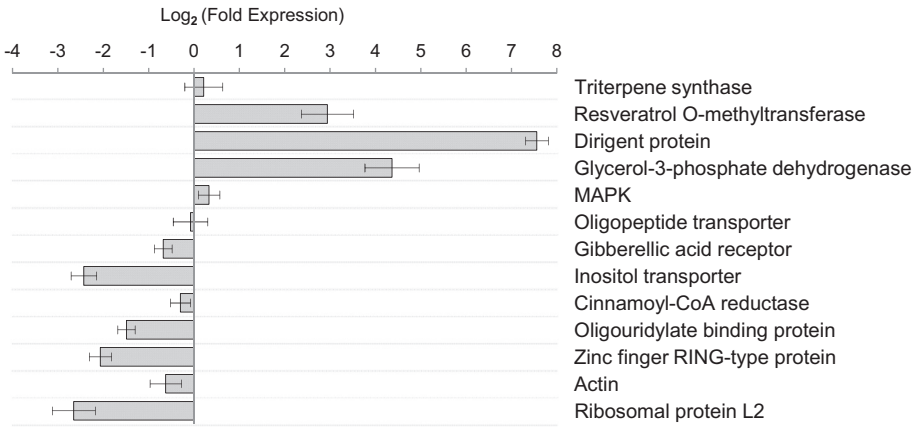

Fig. 2. qRT-PCR analysis of the SSH-detected transcripts and two commonly used normalization genes (Actin, L2 ribosomal protein). Relative gene expression quantification between symptomatic and asymptomatic grapevine leaves was calculated based on triplicated PCR reactions from four biological replicates, using *UBC* and *VAG1* as normalization genes.

[26]. Given the substantial *DIR* upregulation in *E. necator*-infected symptomatic grapevine leaves, a transcriptomic approach was applied to analyze the "metabolic neighbourhood" directly concerned with, or centred in coniferyl alcohol (Fig. 4). The metabolic pathway information was obtained through KEGG database [27,28]. The main objective was to investigate whether the differential transcription observed for SSH-identified *DIR* was consistent with the ones from the remaining branching genes, regarding the reported coniferyl alcohol metabolic fate (i.e. yield of (+)-pinoresinol). According to the phenylpropanoid metabolic pathway described for several plant species, which is illustrated in Fig. 4, a study on the relative expression of the genes encoding caffeic acid *O*-methyltransferase (*CaOMT*), coniferyl-alcohol dehydrogenase (*CAD*), ferulate-5-hydroxylase (*F5H*), coniferyl-alcohol glucosyltransferase (*UDP-GT*), coniferin beta-glucosidase (*ConGlu*) and pinoresinol reductase (*PRR*) was conducted. The *V. vinifera* genome database was searched for each of the previous gene orthologues and PCR primers were designed (Table 2) within the conserved regions of each orthologue family. Melting curve analysis for both *UDP-GT* and *ConGlu* amplicons consistently revealed non-specific amplifications, a result which did not allow data gathering concerning the interconversion between coniferyl-alcohol and coniferin. Relative transcription $\log_2$(fold values) determined for the remaining coniferyl-alcohol branching genes is shown in Fig. 5.

3. Discussion

3.1. SSH cDNA libraries

In contrast to conventional transcriptomic studies, regarding plant–pathogen interactions, where the experimental design is set to identify early or midterm host responses induced by the pathogen, the experimental approach followed in the present work emphasizes long term transcriptional modifications. Moreover, it focuses on differential expression patterns occurring within the host tissues (*V. vinifera* leaves) due to prolonged and successful fungal colonization when compared to tissues that, although having been exposed to *E. necator* spores, remain asymptomatic. PAMP-triggered immunity (PTI) and systemic acquired resistance (SAR) constitute key elements in plant defence mechanisms [29]. However, PTI relies on the recognition of highly conserved epitopes among pathogens and thus, comprises, along with pre-formed chemical and physical barriers, a nonhost-resistance mechanism [30]. Conversely, SAR is based on much higher degree of specificity towards pathogens and is mediated by the products of disease resistance (*R*) genes after the plant is locally infected by the pathogen [31]. Nevertheless, apart from the initial recognition process, both PTI and SAR seem to share downstream signalling machinery

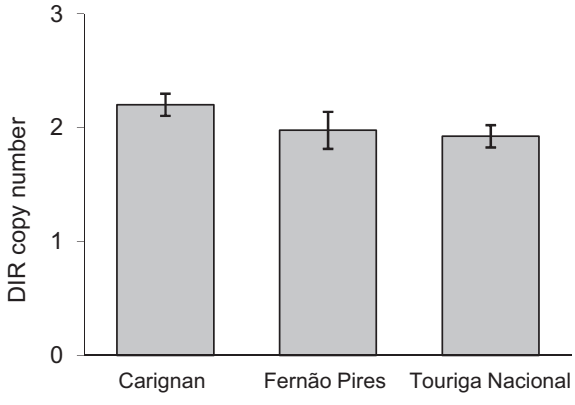

Fig. 3. Genomic *DIR* copy number from grapevine cultivars with different powdery mildew susceptibilities. Absolute copy number was determined through qPCR using the relative quantification method where the genomic DNA of Pinot Noir was used as calibrator. Three Pinot Noir single-copy genes were used for normalization.

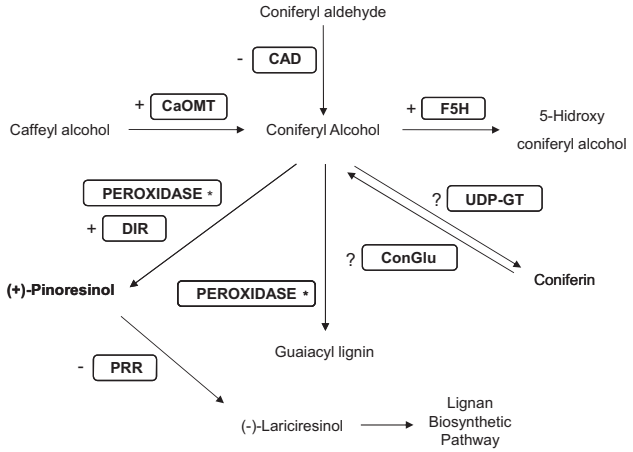

Fig. 4. Coniferyl alcohol branching enzymes. Overview of *V. vinifera* genes directly involved in coniferyl alcohol metabolism and their determined up (+)/down-regulation(−) in *E. necator* symptomatic leaves.

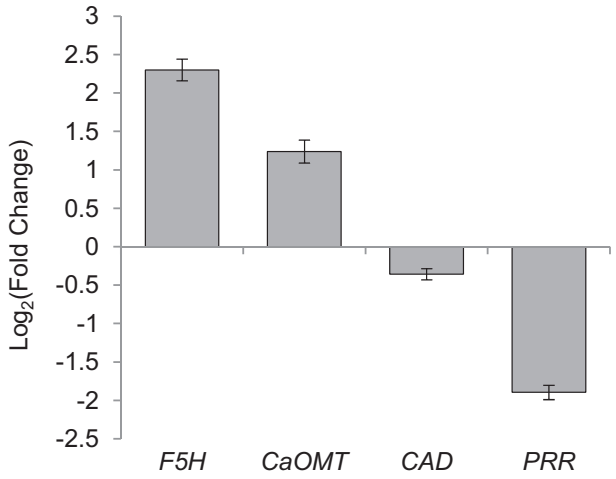

Fig. 5. qRT-PCR analysis of the coniferyl alcohol related genes. Relative gene expression quantification between symptomatic and asymptomatic grapevine leaves was calculated based on triplicated PCR reactions from two biological replicates, using *UBC* and *VAG1* as normalization genes.

and essentially differ from one another by the extent of the pathogen-induced responses, transient in PTI and prolonged in SAR [32].

By applying the SSH technique to determine differentially expressed genes between symptomatic (S) and asymptomatic (A) leaves from powdery mildew infected grapevine (*V. vinifera*, cv. Touriga Nacional), the genes exclusively involved in PTI are expected to be overlooked. Given that, in this study, S and A samples were both collected from plants exposed to the same initial external stimulus and subsequently grown for 30 days with generalized powdery mildew infection, it is reasonable to assume that at least some of the detected transcript changes will be a consequence of an activated SAR mechanism. Moreover, the detection of mRNA variations reflecting host attempts to maintain homoeostasis is expected while excessive nutrient consumption is occurring due to the pathogen presence. In addition, the pathogen also exerts pressure to modulate the host gene expression to its own benefice. In other words, this experimental approach accounts for the permanent host genetic manipulation by the pathogen to fulfil its nutritional needs and to suppress the host specific recognition mechanisms. Anyhow, without additional data, the SSH-detected transcripts (Table 1) can only be assigned to standard functional classes: signalling processes and secondary metabolite biosynthetic pathways, which for the most part concern genes associated with defence-related mechanisms.

The apparently reduced number of SSH-identified genes may be tentatively explained by the premise that the cDNA library screening process, namely the use of non-radioactive labelled probes, did not show enough sensitivity to discriminate between the individual relative transcript abundance of both cDNA subtraction pools. Thus, despite the positive evidence on the subtraction efficiency, an unknown number of the overall "subtracted" transcripts may not have been detected in this study.

3.2. Validation of reference genes for qRT-PCR studies

qRT-PCR is currently one of the most powerful and sensitive techniques for the rapid and reliable quantification of gene transcript levels. Yet its accuracy relies heavily on appropriate normalization procedures to compensate for sample differences such as initial amount, RNA integrity, enzymatic efficiencies, and differences in the overall transcriptional activity of the tissues under analysis [33]. In recent years it became clear that the traditional housekeeping genes used for normalization in northern blots or semi-quantitative RT-PCR were not suitable enough when higher sensitivity techniques are used [34]. Nevertheless, to date, many gene expression studies have been published without an appropriate reference gene selection. In the present study, the need to accurately validate the internal controls used in qRT-PCR for each experimental condition tested was further emphasized. A set of six commonly used reference genes was tested for expression stability under the experimental conditions analyzed in this study using the geNorm algorithm. The candidate reference gene selection criteria must account for potential co-regulation between candidates, since their stability assessment relies on the principle that the expression ratio between two ideal reference genes is identical in all samples. Therefore, identically regulated genes tend to be top ranked in geNorm even if their expression levels fluctuate considerably among samples [25]. Accordingly, and to address this issue, the reference gene candidates were chosen on the basis of their participation in distinct cellular metabolic pathways: ribosomal protein L2 [34], elongation factor 1 alpha [33], cyclophilin [23], phosphoenolpyruvate carboxylase [35], ubiquitin conjugating enzyme [36] and vacuolar ATP synthase subunit G [24].

Reasonably, given the nature of the host–pathogen interaction under study, the evaluation of other commonly used housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase, actin or tubulin, was not considered. Biotrophic pathogens, like *E. necator*, are known for their essential and highly specialized infection structures, the haustoria [9]. Among other functions, haustoria are responsible for the nutrient supply of the pathogen, uptaking amino acids and monosaccharides from the host cells [37]. Hence, the pathogen is expected to directly and greatly affect carbohydrate pools in the host, making genes encoding glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase highly likely targets for gene transcriptional modulation and therefore unsuitable to be used as reference genes in qRT-PCR. For similar reasons, actin and tubulin were not within the group selected for candidate reference genes. Plant cytoskeleton is well known to play an important role in defence against pathogens, with several studies reporting actin and tubulin rearrangements in the host upon pathogen attack [38].

To become aware of the possible bias magnitude generated by the use of an inadequate reference gene selection, the relative transcription levels of the genes encoding actin and the ribosomal protein L2 were measured. The poor stability of the former had already been shown for biotic stress in potato [23], whereas the latter was shown to be the worst ranked candidate in the present study. Both actin and ribosomal protein L2 are downregulated in symptomatic leaves (when compared to asymptomatic ones), with approximate fold-change values of −1.5 and −6.3, respectively (Fig. 2). These data confirm the study of Derveaux and colleagues, who reported that small changes in expression levels should not be based on single non-validated reference genes to prevent significantly biased results [39].

3.3. Differential gene expression quantification by qRT-PCR

Regarding the SSH-identified transcripts and independently of the relative expression quantification accuracy, one cannot get into profound considerations about their role in plant defence. Still it is possible to correlate our results with others described in the literature as far as plant pathogen interactions are concerned. It was interesting to notice that none of the detected genes had correspondence with the ones detected by Fekete et al. which performed a similar gene expression study during the early stages (up to 48 h)

of *E. necator* infection in grapevine leaves [40]. Such observation highlights the infection process dynamics evidencing distinct host transcriptional responses/modifications during the course of infection. Moreover, within the time lapse Feteke carried his experiments, significant differential expression changes were also observed along the first 48 h of infection. We therefore consider that the present study provides novel and valuable information about *V. vinifera–E. necator* interaction in the sense that it describes transcriptomic changes occurring long after the infection is established and possibly illustrates host manipulation by the pathogen and the systemic acquired resistance mechanism developed by the asymptomatic leaves of the plant.

As mentioned before, the majority of the detected genes in the present study belong to signalling pathways or to secondary metabolite biosynthetic routes. The most relevant genes are discussed.

F1B5 EST (KC748395), coding for a glycerol-3-phosphate dehydrogenase, was found to be upregulated in powdery mildew symptomatic leaves, with a fold-change value of +20.6 when compared to asymptomatic leaves. Glycerol-3-phosphate was recently reported as a regulator of plant defence signalling. Although the mechanisms underlying this signalling pathway remain unexplored, it has been shown that *Arabidopsis thaliana* mutants in glycerol-3-phosphate synthesizing genes, such as glycerol kinase or glycerol-3-phosphate dehydrogenase, display enhanced susceptibility to *Colletotrichum higginsianum*, a hemibiotrophic ascomycete responsible for anthracnose disease in *Brassica* sp. [41].

Clone R2A10 (KC748405), codes for an inositol transporter, which was observed to be downregulated (−5.38 fold change) in symptomatic leaves. Myo-inositol plays an important role as the structural basis for a number of secondary messengers in the signal transduction pathways which modulate intracellular events through free Ca^{2+} level regulation [42]. Though ubiquitous in biochemical pathways, myo-inositol metabolism is regarded to intervene in elicitor-induced phytoalexin production and programmed cell death [43,44]. This makes sense as part of the overall fungal strategy in keeping the host defences low during the long-time it operates as a parasite of grapevine leaves.

R2C3 EST (KC748410), codes for a hormone-sensitive lipase (downregulated: −1.60 fold change) with high homology for a gibberellic acid receptor. Besides playing an important role in plant growth and development, gibberellic acid hormonal signalling is also involved in plant defence mechanisms. Moreover, it has been reported as a potential manipulation target in host–microbe compatible interactions [45].

Concerning clone F1D5 (KC748408), coding for a mitogen-activated protein kinase (MAPK), a slight powdery mildew-induced upregulation (+1.26 fold change) was observed. MAPK cascade-mediated signalling is an essential step in the establishment of resistance to pathogens [4]. Within the MAPK family, F1D5 displays the highest homology with MAPK4, which negatively regulates biotic stress signalling, namely systemic acquired resistance [46].

According to our results, R1C7 transcript (KC748397), highly similar to cinnamoyl-CoA reductase (CCR), was weakly repressed in symptomatic leaves. Being a key enzyme in lignin biosynthetic pathway, its regulation directly affects lignin deposition patterns in plants [47]. Several genes encoding CCR have already been shown to be associated to both developmental stages and biotic/abiotic stresses [47–49]. The lignification process can be an important step towards pathogen resistance, either through lignin deposition or formation of lignin-like compounds [50,51]. Thus, the observed CCR downregulation, in this case, can hypothetically be perceived as host manipulation by the pathogen.

An overexpression in symptomatic leaves regarding clone F1C5 (triterpene synthase), F1A11 (resveratrol-*O*-methyltransferase) and F1E6 (DIR) was also observed. The corresponding genes are directly involved in the synthesis of secondary metabolites exhibiting antimicrobial properties. Triterpene synthases are oxidosqualene cyclases catalyzing the cyclization of 2,3-oxidosqualene to afford triterpenoid compounds such as lupeol, betulinic acid or beta-amyrin [52,53]. The SSH-detected oxidosqualene cyclase (KC748400) has the highest homology with beta-amyrin synthase. *trans*-Resveratrol, a trihydroxystilbene phytoalexin is, *per se*, an antifungal compound reported to be produced in plants in response to pathogen attack [54]. Nevertheless, subsequent *trans*-resveratrol metabolic modifications, like dimerization or *O*-methylation, can yield products with enhanced antifungal activity [55]. *O*-Methylation of *trans*-resveratrol to afford pterostilbene, a highly toxic metabolite, is catalyzed by resveratrol-*O*-methyltransferase, whose expression was observed to increase in grapevine leaves upon downy mildew infection [56]. This correlates well with the present work where the SSH-detected resveratrol-*O*-methyltransferase (KC748406) is upregulated in grapevine leaves (+7.68 fold change) due to *E. necator* colonization. Dirigent proteins, although devoid of enzymatic activity, play an important role as chiral auxiliaries to direct the stereochemistry of the reactions in which they participate [15,20]. Up to date, most of the functionally characterized dirigent proteins were shown to participate in lignan biosynthesis, namely in the dimerization of coniferyl alcohol to afford (+)-pinoresinol, a key step in lignan biosynthesis [16]. Lignans constitute an abundant class of phenylpropanoid dimers, well recognized for their antifungal properties [57,58]. Among the SSH-detected transcripts, the gene encoding the dirigent protein (KC748418) displayed by far the highest differential transcription level between symptomatic and asymptomatic leaves from powdery mildew infected grapevine. Its upregulation magnitude in symptomatic leaves reached fold-change values up to 150 times higher than the remaining SSH-detected transcripts, highlighting a great potential relevance in plant defence mechanisms.

3.4. Dirigent proteins

Genes encoding dirigent proteins have been described as being constitutively expressed at very low levels but rapidly induced in response to biotic stress [19,59]. This is consistent with the observed upregulation intensity for the detected dirigent protein gene. Considering the potentially important role of DIR in plant protection against pathogens and the low constitutive expression level of the gene encoding it, the transcriptional rate, and thus the speed at which the plant reacts to stress, could be limited by gene dosage. Therefore, since no data could be gathered about *E. necator*-induced *DIR* transcription in grapevine cultivars with different powdery mildew susceptibilities, *DIR* gene copy number was determined for three *V. vinifera* cultivars (Carignan, Fernão Pires, and Touriga Nacional). All three cultivars were shown to possess an equal number of *DIR* copies in their genome, which was the same as that contained in Pinot Noir. Thus, it is possible to conclude that powdery-mildew susceptibility in *V. vinifera* cultivars is not directly correlated to *DIR* gene copy number, despite the apparent great potential importance played by DIR in plant protection against pathogens.

Considering the genes differentially transcribed between symptomatic and asymptomatic leaves from *E. necator*-infected *V. Vinifera* and the only reaction described so far targeted by dirigent proteins of the DIR-a subfamily (dimerization of coniferyl alcohol to afford (+)-pinoresinol), the transcription levels of the genes whose products operate in the metabolic neighbourhood of coniferyl

alcohol were analyzed (Fig. 4). The main objective of such an experiment was trying to correlate the expression level of those genes involved in the metabolism of the monolignol with the observed, fungal-induced upregulation of the *DIR* gene. Even though this analysis is not representative of the extremely intricate cellular metabolism, it may provide clues indicative of major metabolic flux alterations. When the transcription rate of most enzymes depicted in Fig. 4 was determined under the conditions originating 180 fold upregulation of the *DIR* gene, it was interesting to note that pinoresinol reductase (PRR), described as the enzyme metabolizing the product of the DIR-assisted reaction, undergoes downregulation. Alternative explanations include divergent branching of lignan biosynthetic pathway, in which pinoresinol is converted into metabolites other than lariciresinol, or the involvement of the DIR in the stereochemical control of other reactions.

In the absence of the DIR protein under study, three isomers are formed by dimerization of coniferyl alcohol molecules, in an oxidative reaction which occurs either spontaneously or in the presence of peroxidases [15]: pinoresinol, dehydrodiconiferyl alcohol (DDCA) and guaiacylglycerol 8-*O*-4′-coniferyl ether (GGCE), in the thermodynamic-dependent, relative proportions of 26–28%, 52–57% and 17–19%, respectively. In a different study, Halls confirmed similar proportions as 0.5:1.0:0.3. However, only (+)-pinoresinol is formed when the DIR protein is present [60]. Furthermore, the complete stereoselectivity is preserved as long as the oxidative capacity does not exceed a point where the DIR protein becomes saturated [15].

Knowing that both DDCA [61] and GGCE [62] have been reported as lignin precursors, a different hypothesis may be formulated to explain the >180 fold increase in *DIR* gene transcription in symptomatic grapevine leaves infected with powdery mildew: to ensure that neither of the two isomers other than (+)-pinoresinol is formed under such conditions. If this hypothesis is correct, a low level of DIR during the initial stages of *E. necator* infection may allow lignin biosynthesis to build up physical barriers, whereas a potent, lignan dependent antifungal activity is considered a host priority at later stages of infection.

4. Experimental procedures

4.1. Plant material and growth conditions

Grapevine (*Vitis vinifera* L., cultivar Touriga Nacional) cuttings used in the experiment were collected from Centro Experimental de Pegões, Portugal, and subjected to heartwood disease screening through microbiology assays. The microbiologic screening was performed using the bottom of the cuttings. Thin wood slices were removed from each cutting, surface-sterilized (ethanol, flame and sodium hypochlorite) and then placed in 0.03% (w/v) chloramphenicol-containing PDA medium (five slices per cutting). The plates were incubated at room temperature for a maximum period of one month, during which morphological identification of the microorganisms present in the wood was performed. Diseased cuttings were discarded.

Healthy *V. vinifera* cuttings, with three buds each, were rooted in water and then transferred to soil (1 L pot per plant). Plants were maintained in a growth chamber at 25 °C with a photoperiod of 16 h (480 μmol m^{-2} s^{-1}). After acclimatization, all plants were simultaneously inoculated with *E. necator* by direct contact with naturally infected grapevine leaves. The primary inoculum was collected from a vineyard in Instituto Superior de Agronomia, Lisbon, Portugal and passed to a set of grapevines in greenhouse which provided the experimental inoculum source. Leaves from the greenhouse plants were used to inoculate all the plants in the growth chamber. Plants were allowed to grow with generalized powdery mildew infection for 30 days prior to sample collection. After the infection stage, fully expanded leaves (fourth and fifth positions from the tip of each shoot) with and without *E. necator* infection symptoms (visible mycelia on the upper leaf surface) were randomly harvested and frozen in liquid nitrogen. Biological replicates for symptomatic (S) and asymptomatic (A) conditions were created by pooling four leaves of each condition per sample. All leaves of each pool were collected from different plants from the same experimental setup.

4.2. RNA extraction

Total RNA extraction was performed using the CTAB (hexadecyltrimethyl ammonium bromide) method (Chang et al.), especially suited for high phenolic content material [63]. Leaves were ground in liquid nitrogen, homogenized at 1 g per 20 mL in extraction buffer (2% (w/v) CTAB, 2% (w/v) polyvinylpolypyrrolidone, 2 M NaCl, 100 mM Tris–HCl pH 8.0, 25 mM ethylenediaminetetraacetic acid (EDTA), 2% (v/v) β-mercaptoethanol) and incubated at 65 °C for 10 min. Samples were extracted twice with one volume of chloroform:isoamylic alcohol (24:1, v/v) and centrifuged at 12,000 *g* during 30 min. The recovered aqueous phase was supplemented with ¼ volume of 10 M LiCl and incubated overnight at 4 °C. RNA was collected by centrifugation at 12,000 *g*, 4 °C during 20 min, and resuspended in 1.5 mL of prewarmed (37 °C) SSTE buffer (0.5% w/v sodium dodecyl sulphate (SDS), 1 M NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA). Each sample was divided in two and again extracted with one volume of chloroform:isoamylic alcohol (24:1, v/v) followed by centrifugation at 15,000 *g* during 10 min. The recovered supernatant was supplemented with 2.5 volumes of ethanol and incubated for 2 h at −20 °C. RNA was precipitated by centrifugation at 4 °C, 15,000 *g* during 30 min. The *pellet* was washed with 70% (v/v) ethanol and resuspended in water. Prior to RT-PCR, samples were treated with RQ1 RNase-Free Dnase (Promega) according to the manufacturer's protocol.

4.3. Suppression subtractive hybridization libraries

Each sample, S and A cDNAs, used to perform SSH was synthesized from 4 μg of total RNA, using the BD SMART™ PCR cDNA Synthesis Kit (Clontech). Following PCR cycle optimization for each sample, both cDNA templates were amplified by LD-PCR through 23 temperature cycles according to the manufacturer's protocol. SSH was performed using the PCR-Select cDNA Subtraction Kit (Clontech) following the manufacturer's instructions. Both S and A samples were used as tester and driver for forward and reverse subtractions, respectively. SSH cDNA pools were cloned and transformed using pCR2.1 vector and TOP10 chemically competent *Escherichia coli* from TA Cloning Kit (Invitrogen). Transformed cells were plated on LB agar plates containing kanamycin (50 μg mL^{-1}) overlaid with X-Gal (40 μL of 40 mg mL^{-1}).

4.4. Dot-blot library screening

White colonies from both subtractions were randomly picked from the libraries, transferred to liquid LB (50 μg mL^{-1} kanamycin) in 96-well plates and incubated overnight at 37 °C with agitation. cDNA inserts from each colony were amplified directly from 1 μL of the corresponding liquid culture in a 20 μL PCR reaction using the Advantage cDNA PCR Kit & Polymerase Mix (Clontech). Reaction mixtures were prepared with the primers Nested Primer 1 (5′ TCGAGCGGCCGCCCGGGCAGGT 3′) and Nested Primer 2R (5′ AGCGTGGTCGCGGCCGAGGT 3′), complementary to the adaptors used in the subtraction process. Thermal cycling was performed

using the following parameters: initial denaturation step at 94 °C for 30 s, 23 cycles at 95 °C for 10 s and 68 °C for 3 min. After agarose gel analysis, dot-blot arrays were prepared by spotting each amplified insert in duplicate into separate nylon membranes. Probing of the cDNA arrays was performed as described in DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) using labelled forward and reverse SSH cDNA pools as probes. Clones displaying differential hybridization between forward and reverse probes were interpreted as differentially expressed and selected for insert sequencing.

4.5. Genomic DNA extraction

Grapevine leaves (1 cm^2 per sample) were ground in liquid nitrogen and homogenized in 1.5 mL tubes containing 300 μL of extraction buffer (0.35 M sorbitol, 0.1 M Tris–HCl pH 8.2, 5 mM EDTA, 2% (v/v) β-mercaptoethanol). Homogenates were supplemented with 300 μL of nuclei lysis buffer (2% (m/v) CTAB, 2 M NaCl, 0.2 M Tris–HCl pH 7.5, 50 mM EDTA) and 120 μL 5% (w/v) Sarkosil, vigorously shaken and incubated at 65 °C for 15 min. Samples were extracted with 600 μL of chloroform:isoamylic alcohol (24:1) and centrifuged at 12,000 *g* during 10 min. Ice-cold isopropanol (0.65 vol.) was added to the recovered aqueous phases and the tubes were gently inverted several times. Nucleic acids were collected through centrifugation at 12,000 *g* during 5 min, resuspended in 100 μL of RNase containing buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA) and incubated for 15 min at room temperature. Samples were again extracted with chloroform:isoamylic alcohol (24:1) and precipitated with isopropanol. Following 70% (v/v) ethanol washing, the pellet was resuspended in 50 μL TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA).

4.6. Real-time PCR

Reverse transcription reactions for gene expression studies were performed using ThermoScript RT-PCR System (Invitrogen) as described by the manufacturer. cDNA was synthesized from 1.5 μg of total RNA and oligo$(dT)_{20}$ primed. RT reactions were carried at 55 °C for 60 min.

All PCR primers were designed with Beacon Designer software (Premier Biosoft International) to target amplicons between 80 and 300 bps. qPCR was performed with iQ SYBR Green supermix (Bio-Rad) using iCycler equipment (Bio-Rad). cDNA for reference gene validation and gene expression studies was diluted to 15–30 ng/μL. Genomic DNA to determine *DIR* copy number was diluted to 10 ng/μL. Reaction mixtures (20 μL) were prepared according to the following methodology: 1 μL of the diluted template, 1 μL primer mix (10 μM each), 10 μL iQ SYBR Green supermix (Bio-Rad), 8 μL H_2O. Thermal cycling was composed of an initial denaturation step for 3 min at 95 °C, 40 cycles at 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. All reactions were performed in triplicate and amplification specificity was assessed through melting curve analysis.

Reference gene validation was performed using geNorm software to determine which of the candidates were most stable under the experimental conditions. Validation was based on amplification efficiency corrected data referring to normalized quantities of 6 candidate reference genes in 8 biological samples. Following geNorm stability ranking, relative gene expression analysis was performed using *UBC* and *VAG1* as normalization genes. The experiments were carried out using four biological replicates for SSH-detected transcripts and two biological replicates for coniferyl alcohol branching enzymes. All reactions were performed in triplicate and amplification specificity was assessed through melting curve analysis.

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