

RESEARCH ARTICLE

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Whole genome wide expression profiles of *Vitis amurensis* grape responding to downy mildew by using Solexa sequencing technology

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Abstract

Background: Downy mildew (DM), caused by pathogen *Plasmopara viticola* (PV) is the single most damaging disease of grapes (*Vitis* L.) worldwide. However, the mechanisms of the disease development in grapes are poorly understood. A method for estimating gene expression levels using Solexa sequencing of Type I restriction-
endonuclease-generated cDNA fragments was used for deep sequencing the transcriptomes resulting from PV infected leaves of *Vitis amurensis* Rupr. cv. Zuoshan-1. Our goal is to identify genes that are involved in resistance to grape DM disease.

Results: Approximately 8.5 million (M) 21-nt cDNA tags were sequenced in the cDNA library derived from PV pathogen-infected leaves, and about 7.5 M were sequenced from the cDNA library constructed from the control leaves. When annotated, a total of 15,249 putative genes were identified from the Solexa sequencing tags for the infection (INF) library and 14,549 for the control (CON) library. Comparative analysis between these two cDNA libraries showed about 0.9% of the unique tags increased by at least five-fold, and about 0.6% of the unique tags decreased more than five-fold in infected leaves, while 98.5% of the unique tags showed less than five-fold difference between the two samples. The expression levels of 12 differentially expressed genes were confirmed by Real-time RT-PCR and the trends observed agreed well with the Solexa expression profiles, although the degree of change was lower in amplitude. After pathway enrichment analysis, a set of significantly enriched pathways were identified for the differentially expressed genes (DEGs), which associated with ribosome structure, photosynthesis, amino acid and sugar metabolism.

Conclusions: This study presented a series of candidate genes and pathways that may contribute to DM resistance in grapes, and illustrated that the Solexa-based tag-sequencing approach was a powerful tool for gene expression comparison between control and treated samples.

Background

Downy mildew of grapes occurs in most parts of the world where grapes are grown, but favors those regions that experience warm, wet conditions during the vegetative growth of the vine. A major outbreak of the disease can cause severe losses in yield and berry quality. Symptoms of DM are usually first noticed on leaves as yellowish and later oily lesions on the leaf's upper surface with a 'downy' mass observed on the corresponding

underside of the leaf. It can also cause deformation of shoots, tendrils, inflorescences and clusters of young berries. Berries become less susceptible as they mature, however rachis infection can spread into the older fruit which leads to direct crop loss by shelling of berries [1].

Downy mildew is caused by the pathogen *Plasmopara viticola* (PV). Primary infection begins with the overwintering oospore on infected leaves or plant litter in the soil that germinates in the spring and produces a sporangium [2]. When plant parts are covered with a film of moisture from rain or irrigation, the sporangium releases small swimming spores (zoospores) that are then spread by splashing water. The spores can germinate by producing a germ tube that enters the green

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tissue (including leaves, inflorescences, bunches and young berries) through the stomates [3]. Secondary infection, which is the major source of disease spread, produces spores that may be mobilized by wind and rain to establish new infection sites. The cycle ends with the sexual production of over-wintering oospores [2].

Different genotypes of grapes show varying level of resistance to PV, ranging from susceptible *V. vinifera*, to the moderately resistant *V. rupestris* and *V. amurensis*, *V. cinerea*, *V. riparia* and *V. candicans*, to the totally resistant *Muscadinia rotundifolia* [4-6]. The world-wide grape industry relies predominantly on *V. vinifera*, which requires chemical protection to produce healthy fruits. However, such chemicals may have negative environmental impacts and/or pose risk to human health. A promising alternative strategy that could simultaneously improve grape health and limit chemical use is to identify the unique genes or mechanisms from resistant species that could potentially confer resistance to the pathogen or lower presentation of symptoms. These elements may potentially be introduced into *V. vinifera* through long-term breeding efforts or transgenic methods. With this perspective, it is important to unravel the molecular basis of natural defense responses in resistant grapevines to DM challenge, including identification of the genetic processes that may contribute to resistance.

Responses to PV have been characterized in various resistant species. Mechanisms of resistance include induction of chemical barriers, initiation of processes that delay invasive growth of mycelia, or mechanisms that establish hypersensitive response after inoculation of PV [7-9]. Genetic and gene expression profiling studies have concluded that *Rpv1*, NPR1 homologs, and PR protein encoding genes contribute to the function of DM resistance in grapevines [10-12]. Others factors, including the amino acid beta-aminobutyric acid [13], and the proteins beta-1, 3-Glucanase [14], stilbene synthase (STS) [15], phenylalanine ammonia lyase (PAL) [16], thaumatin-like proteins and chitinase [17] may also play an important role in DM resistance. Many attempts, including transgenic [18-21] and traditional breeding approaches [10,22,23], have been undertaken to introgress resistance into *V. vinifera* genotypes.

To understand the mechanism(s) of the host resistance at the molecular level, a critical first step is to identify the transcripts that accumulate in response to the pathogen attack. In this study, "Zuoshan-1", a clonal selection from wild *V. amurensis* with cold hardiness and high resistance to DM [24], was employed to identify a set of candidate genes associated with DM resistance using Solexa sequencing technology. Solexa sequencing is a technology capable of obtaining novel information for whole-genome-wide transcript expression without prior

sequence knowledge. This report presents the finding of these tests.

Results

Inoculation and symptom development

The fourth unfolded leaf from the shoot apex of "Zuoshan-1" was inoculated with PV. No visible symptoms were observed in the first 4 days (Figure 1a and 1b). The 'downy' mass was obviously observed on the 6th day (Figure 1c) and exacerbated on the 8th day (Figure 1d). Oil spots emerged gradually on the site of pathogen and the spores did not spread to the other healthy tissues 18 days after inoculation (Figure 1e and 1f).

Tag identification and quantification

A total of 8,549,948 and 7,527,499 tags were sequenced in infected (INF) and control (CON) libraries, respectively (Table 1). After filtering out low quality tags (tags containing 'N' and adaptor sequences), 8,474,583 and 7,525,307 tags (noted herein as "clean" tags) remained in INF and CON libraries. To increase the robustness of the approach, single-copy tags in the two libraries (247,900 in INF and 253,156 in CON library) were excluded from further analysis. As a result, a total of 8,226,683 and 7,272,151 clean tags remained from the two libraries, from which 233,653 (INF) and 203,514 (CON) unique tags were obtained. There were 30,139 more unique tags in the INF than in the CON library, possibly representing genes related to pathogen interaction and symptom development. The percentage of unique tags rapidly declined as copy number increased, indicating only a small portion of the transcripts were expressed at high level in the conditions tested.

Depth of sampling

Saturation of the library is determined by identification of unique tags. Sequencing reaches saturation when no new unique tags are detected. The results shown in Figure 2 indicate that INF and CON libraries were sequenced to saturation, producing a full representation of the transcripts in the conditions tested. In both libraries fewer unique tags were identified as the number of sequencing tags increases, reaching a plateau shortly after 6 M tags were sequenced. No new unique tags were identified as the total tag number approached 8.5 M in INF library and 7.5 M in CON library.

Annotation analysis of the unique tag

The unique tags were compared against the genome and gene sequences of *V. vinifera* cv. Pinot Noir [25] using blastn. Tags with a complete match or one base pair mismatch were considered further. The results in Table 2 show that a substantial proportion of tags



Figure 1 Symptom development on leaf surface of “Zuoshan-1” after PV infection. The fourth unfolded leaf from the shoot apex of “Zuoshan-1” was inoculated on (a) day 0. Subsequent images depict the state of infection and symptom development on (b) day 4, (c) day 6, (d) day 8 and (e and f) 18 d. Panel e shows the upper leaf and panel f shows the lower leaf surface.

(81.60% in INF library and 83.72% in CON library) matched to the “Pinot Noir” genome, and 91,638 (39.21% of unique tags) and 83,079 (40.82% of unique tags) in INF and CON library matched to 18,841 (61.91%) and 18,068 (59.37%) “Pinot Noir” genes. Further analysis revealed that 82,886 unique tags (35.47%) in INF library and 75,290 (36.99%) in CON library matched to only one gene sequence in the “Pinot Noir” genome (Table 2). These data indicated that

Table 1 Solexa tags in the infected (INF) and control (CON) libraries

	INF	CON
total tag	8549948	7527499
clean tag	8474583	7525307
clean tag copy number = 1	247900	253156
unique tag	233653	203514
unique tag copy number >5	98318	80345
unique tag copy number >10	63202	51438
unique tag copy number >20	39772	31441
unique tag copy number >50	19776	14804
unique tag copy number >100	10615	7701

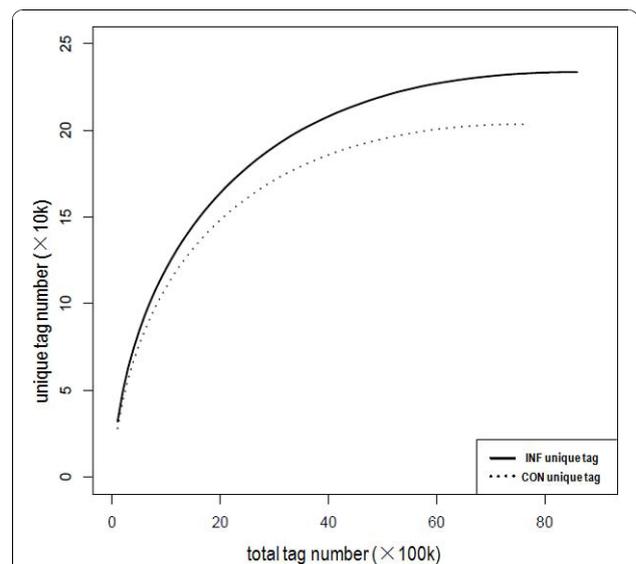


Figure 2 Accumulation of Solexa total tag and unique tag in the two libraries. New unique tag (“y” axis) of INF (solid line) and CON (broken line) libraries decreased as the solexa sequencing increased (“x” axis). The total unique tag was 233,653 in INF and 203,514 in CON library.

Table 2 Annotation of "Zuoshan-1" Solexa tags against the "Pinot Noir" genomic sequence

	INF		CON	
	match to genome	match to gene	match to genome	match to gene
unique tag	190665 (81.60%)*	91638 (39.21%)*	170380 (83.72%)*	83079 (40.82%)*
matched genes		18841 (61.91%)#		18068 (59.37%)#
unique tag matched to one gene		82886 (35.47%)*		75290 (36.99%)*
matched genes		15249 (50.51%)#		14549 (47.81%)#

Note: *percentage of matched tags/total tags;#percentage of matched genes/total assembled CDs of "Pinot Noir".

approximately 50% of transcripts predicted in grape are expressed in the infected or control leaves, with more transcripts present in the infected sample.

Tags with no homology to grape were compared with blastn to the VBI Microbial Database [26] containing genomic sequence information from *Phytophthora sojae*, *Phytophthora infestans* and *Hyaloperonospora parasitica*. There were 251 tags identified in INF library found to be identical to those of the oomycete during PV infection (additional file 1).

Comparison of gene expression level between the two libraries

Differences of tag frequencies that appeared in the INF and CON libraries were used for estimating gene expression levels in response to PV infection. The transcripts detected with at least two-fold differences in the two libraries are shown in Figure 3 (FDR <0.001). The red dots (3,125) and green dots (1,847) represent transcripts higher or lower in abundance for more than two fold in INF library, respectively. The blue dots represent transcripts that differed less than two fold between the two libraries, which were arbitrarily designated as "no difference in expression". The DEGs with five fold or greater differences in accumulation were shown in Figure 4. A total of 513 genes (about 0.9% total unique tags) increased by at least five fold, and 167 genes (about 0.6% total unique tags) were decreased by at least five fold in the INF library, while the expression level of 98.5% unique tags was within five-fold difference between the two samples.

Of DEGs with differences greater than twenty fold (Table 3), 69 genes were present at higher levels in the INF library, 67 of which were associated with defense (6), transport (3), transcription (11), signal transduction (14) and metabolism (33). The highest DEG was phosphate-induced protein gene which was present at 229 fold of control levels. Among these highly expressed genes, many were associated with senescence, abiotic and biotic stresses.

Fifteen DEGs were less abundant in the INF library. Those present twenty fold or more in the CON library were also listed in Table 3, in which 13 genes were classified as defense (2) and metabolism (11), including genes encoding cytochrome P450 and PR proteins. The

greatest differences between INF and CON DEGs were (-)-germacrene D synthase and immunoglobulin/major histocompatibility complex that both were present 164-fold lower in the INF library than in the CON library.

Real-time RT-PCR analysis

In order to validate Solexa expression profiles, the steady-state transcript levels of 12 "defense related" genes were analyzed. Among them, seven genes (CHI4D, TL3, PR10, TIP2;1, CYSP, ERF4, STS5) were upregulated and five genes (THX, SHM1, HypP, GLO, ClpP) were downregulated (Figure 5). Actin, tested to be stable in our previous work, was chosen as a reference gene for data normalization. The trend of RT-PCR based expression profiles among these selected genes was similar to those detected by Solexa-sequencing

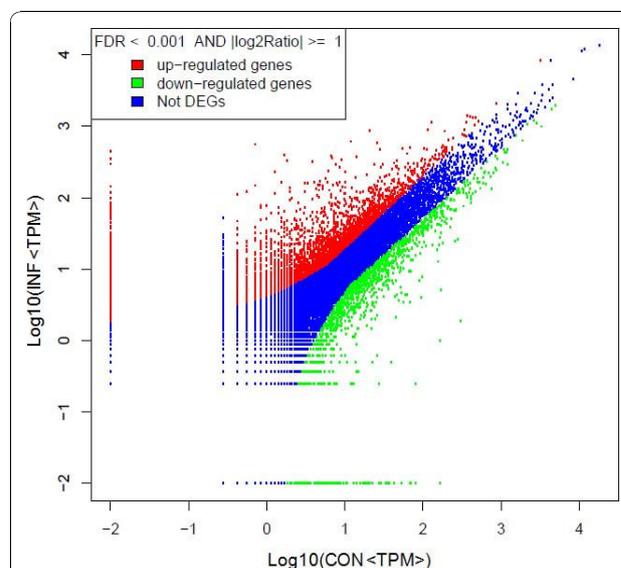
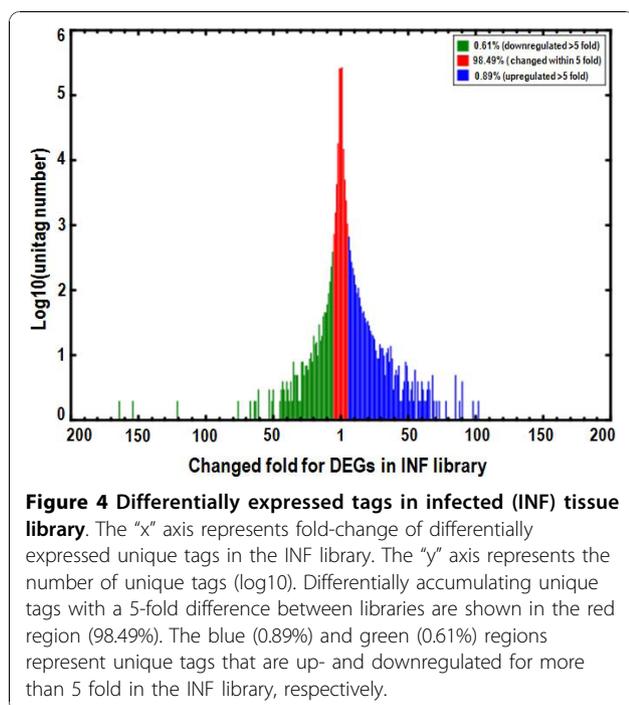


Figure 3 Comparison of gene expression level between the two libraries. For comparing gene expression level between the two libraries, each library was normalized to 1 million tags. Red dots represent transcripts more prevalent in the infected leaf library, green dots show those present at a lower frequency in the infected tissue and blue dots indicate transcripts that did not change significantly. The parameters "FDR <0.001" and "log₂ Ratio ≥ 1" were used as the threshold to judge the significance of gene expression difference.



based method. However, the scales of difference between the INF and CON were generally smaller in Real-time PCR (1-18 fold differences) than in those detected by the Solexa-sequencing based method (2 - 57 folds) (Table 4).

Pathway enrichment analysis of DEGs

The PV affected biological pathways were evaluated by enrichment analysis of DEGs. Significantly enriched metabolic pathways and signal transduction pathways were identified. A total of 115 pathways were affected by up- and 107 were affected by down-regulated DEGs, respectively (additional file 2 and 3). DEGs with pathway annotation were listed according to enrichment priority (additional file 4 and 5). The first ten enriched pathways were reported in Table 5. Pathways with Q value < 0.05 are significantly enriched.

Ribosomal-associated proteins constituted the only significantly affected pathway for the upregulated DEGs (Q < 0.05). Other non-significant enriched pathways with large number of upregulated DEGs included amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, secondary metabolism, plant hormone biosynthesis, and spliceosome associated proteins. There were more significantly enriched pathways (10) for the down-regulated DEGs, which were involved in photosynthesis, as well as metabolism of folate, nicotinate, nicotinamide, fructose, mannose, pyruvate, polyketide sugar unit, and purines, along with alkaloids from histidine and purines.

Discussion

In this report Solexa sequencing technology, a high-throughput DNA sequencing approach, was utilized to estimate gene expression in libraries prepared from infected and control tissues. The results (Figure 2) provided estimates of gene expression as determined by the frequency that any given tag (representing a transcript) is sequenced. The data indicate that there is sufficient coverage depth to reach saturation, that is, a complete assessment of all transcripts present in the libraries. Theoretically, the rate of novel tag discovery should equal zero if all unique tags of the initial sample had been sequenced. However, this number might be slightly higher because new tags may be added due to the accumulation of sequencing errors as the size of the library increased [27]. Strict filtering and conservative matching allows recognition of erroneous tags, which are then disregarded. All of these precepts may contribute to a loss of substantial sequence information. However, loss of some data potentially made the results more conservative, revealing only robust and bona fide differences. Moreover, the total number of tags after stringent filtering was sufficient for annotation to the reference genes in the grape genome sequence. Theoretically, tags should be generated by *NlaIII* from the 3'-most ends of transcripts, but almost 50% of tags from other *NlaIII* sites were also generated in our result. Since only one tag could be generated in each transcript from any *NlaIII* site in a cDNA, these other *NlaIII* tags represented a given gene redundantly in the expression profile. This phenomenon accounts for the inflated number of unique tags generated (about 200,000) relative to that of the annotated grape genome (about 30,000). These other tags may also arise because of alternative splicing or incomplete enzyme digestion.

The results represent the first large-scale investigation of the gene expression in DM analysis of grapevine. Polesani et al [28] reported 804 transcripts identified in PV infected leaves of susceptible cultivar “Riesling” using cDNA-AFLP. Figueiredo et al [29] found 121 transcripts, representing 29 unique gene differentially expressed between two *V. vinifera* cultivars “Regent” and “Trincadeira” (resistant and susceptible to fungi, respectively) by cDNA microarray. In the current study, 15,249 putative genes were identified among the Solexa sequencing tags for the INF library and 14,549 for the CON library.

The steady-state transcript level for a set of selected genes was confirmed by Real-time RT-PCR. Although the differences in gene expression did not match the magnitude of those detected by Solexa-based sequencing method, the trends of up- and down- regulation were similar. The lower expression level detected by Real-time

Table 3 List of DEGs changed for 20 fold and more in INF library

Gene	Annotation	Stress related function	Accession	Identity	Fold
Upregulated genes					
Defence					
GSVIVT00025506001	polygalacturonase-inhibiting protein [<i>Vitis labrusca</i> x <i>Vitis Riparia</i>]	inhibits fungal endopolygalacturonases	ACS16072.1	333/333 (100%)	60
GSVIVT00001105001	thaumatin-like protein [<i>Vitis vinifera</i>]	pathogen defence; drought and heat combination	AAQ10092.1	217/225 (96%)	57
GSVIVT00017370001	harpin-induced protein-related/HIN1-related/harpin-responsive protein-related [<i>Arabidopsis thaliana</i>]	pathogen defence; senescence	NP_565634.1	141/267 (52%)	33
GSVIVT00002965001	TMV response-related protein [<i>Zea mays</i>]	Tobacco Mosaic Virus response	ACG48457.1	39/91 (42%)	32
GSVIVT00005362001	glutaredoxin [<i>Populus trichocarpa</i>]	senescence	EEE75685.1	91/155 (58%)	29
GSVIVT00024683001	beta-glucosidase [<i>Rosa</i> hybrid cultivar]	activation of phytoanticipins	BAG13451.1	382/531 (71%)	21
Transport					
GSVIVT00001094001	multidrug resistance pump, putative [<i>Ricinus communis</i>]	fungal resistance	EEF51093.1	407/509 (79%)	121
GSVIVT00015121001	mitochondrial dicarboxylate carrier protein, putative [<i>Ricinus Communis</i>]	aluminum tolerance	EEF48606.1	271/324 (83%)	38
GSVIVT00030447001	multidrug resistance protein ABC transporter family protein [<i>Populus Trichocarpa</i>]	Senescence; drought and heat combination	EEE80779.1	64/194 (32%)	25
Signal transduction					
GSVIVT00030628001	leucine-rich repeat receptor-like protein kinase [<i>Nicotiana tabacum</i>]	senescence	AAF66615.1	644/923 (69%)	145
GSVIVT00006178001	FERONIA receptor-like kinase [<i>Arabidopsis thaliana</i>]	defence, stresses	ABT18100.1	317/621 (51%)	56
GSVIVT00019504001	MAP3K-like protein kinase [<i>Arabidopsis thaliana</i>]	disease resistance, drought and heat combination	CAB16796.1	184/359 (51%)	52
GSVIVT00002706001	calmodulin-binding protein [<i>Arabidopsis thaliana</i>]	senescence	NP_565379.1	21/45 (46%)	39
GSVIVT00020989001	calcium-binding EF hand family protein [<i>Arabidopsis thaliana</i>]	defence related; senescence; drought and heat combination	NP_568568.1	81/166 (48%)	35
GSVIVT00029809001	ethylene-regulated transcript 2 (ERT2) [<i>Arabidopsis thaliana</i>]	senescence	CAB45883.1	96/204 (47%)	34
GSVIVT00036549001	calmodulin-binding protein [<i>Arabidopsis thaliana</i>]	senescence	NP_565379.1	149/366 (40%)	28
GSVIVT00002973001	calmodulin binding protein-like [<i>Elaeis guineensis</i>]	senescence	ABP04242.1	89/135 (65%)	27
GSVIVT00025017001	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor, putative [<i>Ricinus communis</i>]	disease, cell death	EEF29110.1	415/639 (64%)	26
GSVIVT00000612001	nodulin-like protein [<i>Arabidopsis thaliana</i>]	drought and heat combination	AAC28987.1	397/550 (72%)	23
GSVIVT00033036001	RING-H2 subgroup RHE protein [<i>Populus tremula</i> x <i>Populus alba</i>]	drought and heat combination	AAW33880.1	168/296 (56%)	22
GSVIVT00009150001	PAR-1a [<i>Nicotiana tabacum</i>]	potato virus Y, SAR induce	CAA58733.1	127/178 (71%)	22
GSVIVT00027614001	receptor-protein kinase-like protein [<i>Arabidopsis thaliana</i>]	drought and heat combination	BAA98098.1	632/849 (74%)	20
GSVIVT00030574001	leucine-rich repeat receptor-like protein kinase [<i>Arabidopsis thaliana</i>]	senescence	ACN59244.1	317/611 (51%)	20
Transcription					
GSVIVT00014947001	zinc-finger protein 1 [<i>Datisca glomerata</i>]	defence, stresses	AAD26942.1	144/246 (58%)	60
GSVIVT00016398001	dehydration-responsive element binding protein 3 [<i>Glycine max</i>]	biotic and abiotic stresses	ABB36646.1	116/187 (62%)	52
GSVIVT00007409001	DRE-binding protein 3b [<i>Gossypium hirsutum</i>]	drought and heat combination	ABB45861.1	134/237 (56%)	22
GSVIVT00020131001	basic helix-loop-helix protein [<i>Nicotiana tabacum</i>]	senescence	BAF30984.1	105/228 (46%)	33
GSVIVT00001092001	Dehydration-responsive element-binding protein 1F, putative [<i>Ricinus communis</i>]	phytohormone, pathogen and environmental stresses	EEF51090.1	143/242 (59%)	30
GSVIVT00007410001	CBF4 transcription factor [<i>Vitis vinifera</i>]	cold stress	ABE96792.1	218/218 (100%)	30
GSVIVT00016403001	jasmonate ZIM domain 1 [<i>Catharanthus roseus</i>]	wounding; herbivory; salinity	ACM89457.1	131/275 (47%)	27

Table 3 List of DEGs changed for 20 fold and more in INF library (Continued)

GSVIVT00028041001	AP2 domain class transcription factor [<i>Malus x domestica</i>]	senescence; drought and heat combination	ADE41117.1	172/327 (52%)	26
GSVIVT00027444001	GRAS family transcription factor [<i>Populus trichocarpa</i>]	chitin response	EEE95719.1	446/586 (76%)	26
GSVIVT00006790001	basic helix-loop-helix (bHLH) family protein [<i>Arabidopsis thaliana</i>]	fugal resistance related; senescence	NP_568850.1	152/239 (63%)	21
GSVIVT00002446001	WRKY transcription factor 21 [<i>Populus tomentosa</i> x <i>P. bolleana</i>]	senescence, stresses	ACV92023.1	196/364 (53%)	21
Metabolism					
GSVIVT00015203001	putative phosphate-induced protein [<i>Nicotiana tabacum</i>]	unidentified	BAA33810.1	243/317 (76%)	229
GSVIVT00016518001	salt responsive protein 2 [<i>Solanum lycopersicum</i>]	drought and heat combination	ACG50004.1	309/464 (66%)	165
GSVIVT00024884001	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase [<i>Chimonanthus praecox</i>]	biotic and abiotic stresses	ABU88887.2	191/377 (50%)	97
GSVIVT00024408001	protein-binding protein, putative [<i>Ricinus communis</i>]	unidentified	EEF27653.1	393/605 (64%)	87
GSVIVT00028930001	ubiquitin-protein ligase, putative [<i>Ricinus communis</i>]	senescence	EEF42248.1	357/602 (59%)	72
GSVIVT00014730001	cytochrome P450 [<i>Populus trichocarpa</i>]	senescence; drought and heat combination	EEE73840.1	261/453 (57%)	70
GSVIVT00000988001	9-cis-epoxycarotenoid dioxygenase 1 [<i>Vitis vinifera</i>]	senescence; defence	AAR11193.1	602/610 (98%)	62
GSVIVT00023009001	ATPP2-A2, putative [<i>Ricinus communis</i>]	unidentified	EEF38353.1	114/158 (72%)	56
GSVIVT00014704001	putative integral membrane protein [<i>Cyanotheca</i> sp. CCY0110]	unidentified	EAZ88012.1	53/176 (30%)	51
GSVIVT00018424001	tropinone reductase, putative [<i>Ricinus communis</i>]	senescence; drought and heat combination	EEF38138.1	194/264 (73%)	48
GSVIVT00032938001	aspartic proteinase nepenthesin-1 precursor, putative [<i>Ricinus communis</i>]	phosphorus deficiency; salt stress	EEF29846.1	306/441 (69%)	39
GSVIVT00024072001	protein phosphatase 2c, putative [<i>Ricinus communis</i>]	senescence	EEF41194.1	254/393 (64%)	37
GSVIVT00015200001	putative phosphate-induced protein [<i>Capsicum chinense</i>]	unidentified	BAG16530.1	186/289 (64%)	37
GSVIVT00022245001	f-box family protein [<i>Populus trichocarpa</i>]	senescence	EEE87327.1	139/345 (40%)	37
GSVIVT00016166001	ATP-dependent DNA helicase [<i>Brevibacillus brevis</i>]	DNA repair	BAH41662.1	16/45 (35%)	36
GSVIVT00024387001	nucleic acid binding protein, putative [<i>Ricinus communis</i>]	oxidative; ABA; abiotic stresses	EEF29282.1	102/164 (62%)	34
GSVIVT00024235001	protein phosphatase 2C [<i>Nicotiana tabacum</i>]	senescence	CAC10358.1	257/429 (59%)	34
GSVIVT00035825001	ubiquitin-protein ligase, putative [<i>Ricinus communis</i>]	senescence	EEF40124.1	572/719 (79%)	32
GSVIVT00019233001	TPA: isoflavone reductase-like protein 3 [<i>Vitis vinifera</i>]	putative defence	CAI56332.1	301/319 (94%)	31
GSVIVT00014029001	TPA_exp: cellulose synthase-like D1 [<i>Oryza sativa</i>]	unidentified	DAA01752.1	999/1171 (85%)	31
GSVIVT00007984001	serine acetyltransferase [<i>Nicotiana plumbaginifolia</i>]	oxidative stress	AAR18403.1	179/307 (58%)	30
GSVIVT00036225001	Beta-expansin 1a precursor, putative [<i>Ricinus communis</i>]	osmotic stress	EEF28288.1	207/259 (79%)	27
GSVIVT00017518001	spotted leaf protein, putative [<i>Ricinus communis</i>]	hypersensitive response; cell death; senescence	EEF38265.1	243/402 (60%)	27
GSVIVT00007452001	wound-induced protein WIN2 precursor, putative [<i>Ricinus communis</i>]	antifungal	EEF31100.1	142/197 (72%)	26
GSVIVT00002450001	UDP-glucose:glucosyltransferase [<i>Lycium barbarum</i>]	drought and heat combination	BAG80556.1	293/464 (63%)	24
GSVIVT00036349001	glucose-1-phosphate adenylyltransferase, putative [<i>Ricinus communis</i>]	drought and heat combination	EEF49428.1	412/531 (77%)	24
GSVIVT00028839001	spotted leaf protein, putative [<i>Ricinus communis</i>]	hypersensitive response; cell death; senescence	EEF52025.1	385/674 (57%)	24
GSVIVT00009741001	f-box family protein [<i>Populus trichocarpa</i>]	senescence	EEE86166.1	93/182 (51%)	24
GSVIVT00019669001	galactinol synthase [<i>Solanum lycopersicum</i>]	oxidative stress; drought; salinity; chilling; heat shock	BAH98060.1	231/316 (73%)	24

Table 3 List of DEGs changed for 20 fold and more in INF library (Continued)

GSVIVT00030537001	senescence-associated protein, putative [<i>Medicago truncatula</i>]	Senescence; drought and heat combination	ABD32641.1	99/144 (68%)	23
GSVIVT00001432001	protein phosphatase 2c, putative [<i>Ricinus communis</i>]	senescence; drought and heat combination	EEF34881.1	319/389 (82%)	23
GSVIVT00033193001	galactinol synthase [<i>Capsicum annuum</i>]	oxidative stress; drought; salinity; chilling; heat shock	ABQ44212.1	239/315 (75%)	21
GSVIVT00023109001	ATEXO70H4 (exocyst subunit EXO70 family protein H4); protein binding [<i>Arabidopsis thaliana</i>]	unidentified	NP_187563.1	331/585 (56%)	21
various functions					
GSVIVT00017533001	PREDICTED: hypothetical protein [<i>Vitis vinifera</i>]	unidentified	XP_002279648.1	500/500 (100%)	20
GSVIVT00020834001	CW14 [<i>Arabidopsis thaliana</i>]	unidentified	BAA87958.1	300/533 (56%)	23
Downregulated genes					
Defence					
GSVIVT00016961001	Immunoglobulin/major histocompatibility complex [<i>Medicago truncatula</i>]	disease resistance	ABP03850.1	426/672 (63%)	-164
GSVIVT00014282001	pathogenesis-related like protein [<i>Arabidopsis thaliana</i>]	defence	AAM66077.1	117/215 (54%)	-67
Metabolism					
GSVIVT00027449001	(-)-germacrene D synthase [<i>Vitis vinifera</i>]	wounding; methyl jasmonate	AAS66357.1	500/553 (90%)	-164
GSVIVT00027451001	(-)-germacrene D synthase [<i>Vitis vinifera</i>]	wounding; methyl jasmonate	AAS66357.1	503/557 (90%)	-150
GSVIVT00027450001	(-)-germacrene D synthase [<i>Vitis vinifera</i>]	wounding; methyl jasmonate	AAS66357.1	274/319 (85%)	-53
GSVIVT00027456001	(-)-germacrene D synthase [<i>Vitis vinifera</i>]	wounding; methyl jasmonate	AAS66357.1	454/545 (83%)	-22
GSVIVT00014725001	cytochrome P450 [<i>Populus trichocarpa</i>]	pathogen induced	EEE73840.1	299/511 (58%)	-41
GSVIVT00014727001	cytochrome P450 [<i>Populus trichocarpa</i>]	pathogen induced	EEE73840.1	269/447 (60%)	-35
GSVIVT00007099001	thioredoxin x [<i>Populus trichocarpa</i>]	defence; abiotic stresses, senescence	EEE90516.1	98/117 (83%)	-39
GSVIVT00008711001	beta-cyanoalanine synthase [<i>Betula pendula</i>]	cyanide metabolism	AAN86822.1	311/352 (88%)	-36
GSVIVT00037489001	non-specific lipid transfer protein [<i>Vitis vinifera</i>]	defence related	ABA29446.1	119/119 (100%)	-28
GSVIVT00029445001	expansin [<i>Vitis labrusca x Vitis vinifera</i>]	defence related	BAC66695.1	252/252 (100%)	-22
GSVIVT00006300001	UDP-glucosyltransferase, putative [<i>Ricinus communis</i>]	defence related	EEF47681.1	268/466 (57%)	-22
various functions					
GSVIVT00005678001	male sterility-related protein [<i>Linum usitatissimum</i>]	unidentified	ACA28679.1	260/503 (51%)	-23
GSVIVT00032599001	hypothetical protein [<i>Vitis vinifera</i>]	unidentified	XP_002284962.1	368/368 (100%)	-22

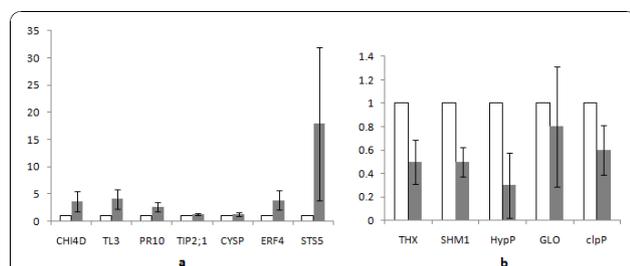


Figure 5 Real-time RT-PCR analysis for twelve differentially expressed genes. Real-time RT-PCR analysis for twelve transcripts in control (white) and infected (gray) samples, including (a) seven more abundant in the INF library and (b) five less prevalent in the INF library as identified by Solexa expression profile. All data were normalized to the actin expression level. Data represent fold change of RQ (relative quantification) in infected vs. control samples. Bars represent RQ standard deviation calculated from three biological replicates.

RT-PCR could be due to the difference of sensitivity between the two technologies. Solexa sequencing has been documented to be more sensitive for estimation of gene expression, especially for low-abundance transcripts compared to microarrays and Real-time RT-PCR [30]. The difference could also be attributed to different inoculation seasons and developmental stages of the grapevines. The materials used for the Solexa sequencing method were obtained from materials inoculated and harvested in September, while materials used for the Real-time RT-PCR analyses were obtained from plants inoculated and harvested in June.

Due to the sensitivity of Solexa sequencing technology, many rare transcripts were detected. Among 536 transcripts present predominantly (<2-20 fold) in the INF library, 89 were not detected in the CON library at all. These genes were predicted to be involved in many plant

Table 4 Genes selected for Real-time RT-PCR

Gene	Description	Forward primer	Reverse primer	Target size	Solexa fold	RT-PCR fold
CHI4D	<i>V. vinifera</i> class IV chitinase (gb AF532966.1)	TCCCACGTTCCCCCTTCT	GTAGCTTGGCTGCCATTTTGTG	59	11	4
TL3	<i>V. vinifera</i> thaumatin-like protein (gb AF532965.1)	ACCCCACTCCAACCATCAAG	GATTTTGCAGAGGCCCATTTG	59	57	4
PR10	Tamnara Tam-RP10 pathogenesis-related protein 10 (dbj AB372561.1)	GGTCAGGCCTCAAGCTATCAA	CAGGGCCTCCGTCTCCTT	56	10	3
TIP2;1	<i>V. vinifera</i> aquaporin TIP2;1 (gb EF364439.1)	GCATCATTTGCACCCATTGC	GCCTGCAGCCAGGATGTT	59	6	1
CYSP	<i>V. vinifera</i> cysteine protease (gb EU280160.1)	CCTCGCAGGAGGAGCACGAT	CCGGCGCAGGTTTGC	54	2	1
ERF4	<i>V. aestivalis</i> putative ethylene response factor 4 (gb AY484580.1)	TCATCACTGCAACTCATCCA	TTACAATCTTCGGCCTCTGA	101	11	4
STS5	<i>V. vinifera</i> stilbene synthase5 (gb AY670312.1)	CGCTCAAGGAGGAAAGACA	AGCCAAACAAAACACCCCAATC	58	12	18
THX	thioredoxin x [Populus trichocarpa] (XP_002310066.1)	TGCTCAGGAATACGGGGACAGA	TCGCGGTTTGCATCAT	61	-39	-2
SHM1	<i>A. thaliana</i> serine hydroxymethyl transferase 1 (ref NM_119954.3)	TGTTTATCAGGTGACCCAGTTT	TGCGTCGAATGCAGCAAGAT	63	-2	-2
HypP	Hypothetical protein LOC100264849	TGCCCTACCTTTGTGACA	GATCAAAATGGCTCATCGGAA	58	-5	-3
GLO	<i>V. pseudoreticulata</i> glyoxal oxidase (gb D201181.1)	TCCCAACGCCGGTATAGC	ACCGTGCCGTAAACGTGTGA	54	-5	-1
ClpP	<i>Carica papaya</i> ATP-dependent Clp protease proteolytic subunit (gb DQ159405.1)	GGGCGCCGGACAAGA	TTTGCAAATCATCCCTAATGGA	55	-2	-2

biological processes, including defense. For example, genes encoding cinnamyl alcohol dehydrogenase, lipase-like protein, glutathione synthetase, GDSL-motif lipase, ankyrin repeat family protein, serine hydrolase, proline-rich cell wall protein and multicopper oxidase were

previously described as plant defense-related genes. Other rare transcripts detected by Solexa technology were predicted to function in signal transduction (protein kinase, calcium ion binding protein, wall-associated kinase), transport (type IIIa membrane protein, ATP

Table 5 List of first ten pathways for up- and downregulated DEGs

Pathway term	Pathway ID	DEGs tested	P value	Q value
Pathways for upregulated DEGs				
Ribosome	ko03010	53 (4.36%)	0.0004	0.0406
Amino sugar and nucleotide sugar metabolism	ko00520	25 (2.06%)	0.0010	0.0563
Glycolysis/Gluconeogenesis	ko00010	28 (2.3%)	0.0043	0.1660
Biosynthesis of alkaloids derived from histidine and purine	ko01065	31 (2.55%)	0.0126	0.3636
Biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid	ko01064	35 (2.88%)	0.0207	0.4459
Starch and sucrose metabolism	ko00500	49 (4.03%)	0.0233	0.4459
Biosynthesis of alkaloids derived from shikimate pathway	ko01063	39 (3.21%)	0.0361	0.5868
N-Glycan biosynthesis	ko00510	10 (0.82%)	0.0528	0.5868
Fructose and mannose metabolism	ko00051	14 (1.15%)	0.0560	0.5868
Selenoamino acid metabolism	ko00450	11 (0.91%)	0.0587	0.5868
Pathways for downregulated DEGs				
Photosynthesis	ko00195	20 (3.14%)	9.9613e-06	0.0011
Photosynthesis - antenna proteins	ko00196	6 (0.94%)	4.2252e-05	0.0023
Folate biosynthesis	ko00790	5 (0.78%)	0.0002	0.0064
Nicotinate and nicotinamide metabolism	ko00760	5 (0.78%)	0.0007	0.0125
Fructose and mannose metabolism	ko00051	13 (2.04%)	0.0007	0.0125
Carbon fixation in photosynthetic organisms	ko00710	13 (2.04%)	0.0007	0.0125
Pyruvate metabolism	ko00620	14 (2.2%)	0.0014	0.0210
Polyketide sugar unit biosynthesis	ko00523	4 (0.63%)	0.0016	0.0210
Purine metabolism	ko00230	21 (3.3%)	0.0018	0.0215
Biosynthesis of alkaloids derived from histidine and purine	ko01065	21 (3.3%)	0.0025	0.0270

binding protein, D-galactonate transporter, peptide transporter), transcription (ccaat-binding transcription factor, AP2/ERF domain-containing transcription factor, mutator-like transposase-like protein), and protein metabolism (ubiquitin-protein ligase, 50S ribosomal protein, S-locus-specific glycoprotein S13 precursor, Rab5-interacting protein). Two novel genes (nectar protein 1, vernalization-insensitive protein) and some genes encoding hypothetical proteins (LOC100244011, LOC100258240, LOC100249110) were also identified from the PV-induced rare DEGs. Among the 608 rare transcripts present more in CON than INF, 69 were not detected at all in the INF library. Most of these transcripts have predicted biological functions in growth regulation (growth regulator protein, A-type cyclin, auxin response factor 8), transport (ATP-binding cassette transporter, AWPM-19-like membrane family protein, copper-transporting atpase p-type), signal transduction (serine-threonine protein kinase, leucine-rich repeat family protein, calcium-binding EF hand family protein, calcium-dependent phospholipid binding), and metabolism (galacturonosyl-transferase 6, methylenetetrahydrofolate dehydrogenase, iron ion binding/oxidoreductase, trehalose-6-phosphate synthase, senescence-associated protein).

Pathway enrichment analysis revealed the most significantly affected pathways during the PV infection in "Zuoshan-1". It is not surprising that the "ribosome-related" pathway was the most affected for the DEGs more common in INF library. This finding implies that the grapevine utilizes new ribosomes or changes in ribosome components to help synthesize additional proteins, such as PR proteins, to protect itself from the pathogen attack. The second affected pathway was the "amino sugar and nucleotide sugar metabolism" pathway. In this pathway genes encoding chitinase were more prevalent in the INF than the CON library. In addition, genes required for cell wall biosynthesis were also affected, such as D-xylan synthase, UDP-glucose dehydrogenase, and UDP-glucose 4,6-dehydratase. These enzymes are involved in the interconversion of nucleotide sugars, and may regulate glycosylation patterns in response to pathogen, thereby linking signaling with primary metabolism and the dynamics of the extracellular matrix. The other noticeable pathways with a large amount of DEGs associated with PV infection were starch and sucrose metabolism, secondary metabolism, plant hormone biosynthesis, and spliceosome-associated proteins. For DEGs less prevalent in infected vs. control libraries, there was significant enrichment for transcripts associated with photosynthesis. This result was similar to the reports of Polesani et al [28,31]. Photosystem I proteins (PsaA, PsaB, PsaC), photosystem II proteins (PsbB, PsbD, PsbO, PsbP, PsbS), cytochrome b6/f complex

(PetD, PetN) and F-type ATPase (beta, alpha, delta, a, b) were all substantially lower in abundance in INF libraries compared to CON libraries. The reduction of photosynthesis was possibly due to the increase of invertase activity in nucleotide sugar metabolism pathway. Invertase would cleave sucrose into hexose sugars and their accumulation inhibits the Calvin cycle.

It was observed that 251 tags identified in INF library were homologous to the oomycete, indicating that they may belong to PV transcripts, predictably noting the presence of the pathogen. Many of these putative PV transcripts corresponded to genes involved in protein metabolism (16S, 18S, 26S, 28S and 60S ribosomal protein subunits) as a requirement for protein synthesis in the pathogen during the plant-pathogen interaction. Many housekeeping genes (alpha-tubulin, elongation factor 1 alpha, ubiquitin and heat shock protein 70) and genes related to immune response (spike 1 protein and cyclophilin) were also detected. Several PV transcripts showed similarity to enzymes involved in carbohydrate and amino acid metabolism (chlorophyll apoprotein, aspartate aminotransferase, glutamine synthetase and hyaluronoglucosaminidase-4), energy production (ATP synthase subunit B, glyceraldehyde-3-phosphate dehydrogenase, phosphoenolpyruvate carboxykinase and nitrate reductase), and cellular transport (transportin 1, K⁺ channel protein and calmodulin).

Transcripts more abundant in infected leaves

A set of transcripts were clearly more abundant in tissue arising after PV infection compared to control. This group possibly contains elements that confer resistance to the spread of the pathogen in "Zuoshan-1". Among these transcripts, those expressed at a relatively high level in infected tissue are of the most interest. These transcripts likely encode genes responding to the pathogen or genuine factors that underlie genetic resistance, which were broadly grouped into the following categories based on their known roles in other plant systems.

Defense response genes

Among defense response genes, thaumatin-like protein [17], polygalacturonase-inhibiting protein (PGIP) [32,33], harpin-induced protein-related [34,35], glutaredoxin [36,37] and beta-glucosidase [38,39] have been widely studied in plant pathogen resistance. Thaumatin-like protein, like many other disease resistant proteins [40], is also induced by abiotic stresses, which may indicate existence of a crosstalk between pathogen and abiotic stresses. In this category, tobacco mosaic virus (TMV) response -related protein (+32 fold in INF vs CON) is associated with TMV attack and may also play an important role in DM resistance of grape.

Transport

Three transcripts were associated with transport function. Multidrug resistance pump proteins (+121 fold in INF vs CON) and multidrug resistance ABC transporter (+25 fold in INF vs CON) are well known transporters in clinical study for bacteria infection of human [41]. Such transporters also have been isolated from plants, such as *Coptis japonica* [42]. They transport several compounds associated with multidrug (antibiotic) resistance which can inhibit pathogen infection in animal model [41,43]. Another gene identified to be transport related is mitochondrial dicarboxylate carrier protein (+38 fold in INF vs CON) which might be involved in the excretion of organic acids and rhizotoxic aluminum tolerance [44].

Signal transduction

There were fourteen transcripts in our results associated with signal transduction. Two came from genes (GSVIVT00030628001, GSVIVT00030574001) encoding leucine-rich repeat receptor-like protein kinases which were more prevalent (145 and 20 fold) in the INF library than in control. Molecules that indicate the presence of pathogen (elicitors) activate host receptors and that rapidly generate an internal signal that triggers early defense responses [45]. Various signals presented in our results, including phytohormones like ABA and ethylene, as well as intracellular messengers like calcium, phosphoinositide and kinases, have been proposed to regulate plant responses in adverse environmental conditions and thus contribute to the coordination of plant stress physiology [46]. Transcripts representing three kinase-encoding genes (GSVIVT00030628001, GSVIVT00006178001, GSVIVT00019504001) were present 52-145 fold higher in INF than CON, and have been widely documented as signaling factors in many stresses [47-50] and senescence [51]. Four transcripts (GSVIVT00002706001, GSVIVT00020989001, GSVIVT00036549001, GSVIVT00002973001) were found to be more abundant (27 to 39 fold) in INF than CON, and were associated with calcium signaling pathway. All of these are also induced by senescence [52] and many stresses [53,54]. Nodulin-like protein (+23 fold in INF vs CON) induced in fungal pathogen treatment [55] and drought/heat combination stress [40] has been shown to be involved in salicylic acid (SA) signaling pathway [56]. A RING-H2 gene (+22 fold in INF vs CON) has demonstrated regulatory function in ABA signaling [57], drought tolerance [57], regulation of growth and defense responses against abiotic/biotic stresses [58]. Ethylene-regulated transcript 2 (ERT2) (+34 fold in INF vs CON) is involved in ethylene response 'circuit' including ethylene synthesis, perception, signal transduction and regulation of gene expression [59]. The PAR-1a (photoassimilate-responsive) protein (+22 fold in INF vs

CON) is a serine/threonine kinase with diverse phosphorylation targets and has been reported to be induced by infection with potato virus Y [60,61].

Transcription

Eleven transcripts associated with transcription were 21 to 60 fold more abundant in INF than CON libraries. Transcripts annotated as zinc-finger protein 1, DREB protein, AP2 domain class transcription factor, basic helix-loop-helix protein, CBF4(C-repeat binding factor 4), jasmonate ZIM domain 1, GRAS family transcription factor, and WRKY transcription factor 21 were all present at higher steady state levels in infected tissue. They have been documented to play important roles in responding to phytohormone stasis, pathogen attack and environmental stresses [62-69].

Metabolism

Synthesis of the hormones

S-adenosyl-L-methionine (GSVIVT00024884001) and 9-cis-epoxycarotenoid dioxygenase 1(NCED1) (GSVIVT00000988001) are transcripts related to synthesis of plant hormones, and were found more frequently (97 and 62 fold, respectively) in the INF library. S-adenosyl-L-methionine is the precursor of ethylene [70] which participates in regulation of growth, development, and responses to stress and pathogen attack in plants [71]. NCED is an important enzyme in synthesizing the phytohormone ABA which plays a central role in responses to pathogen attack [72].

Protein metabolism

Twelve transcripts related to protein metabolism were more abundant in the INF library, 21 fold to 72 fold. Among them, ubiquitin-protein ligase (GSVIVT00-028930001, GSVIVT00035825001), spotted leaf protein (GSVIVT00017518001, GSVIVT00028839001) and f-box family protein (GSVIVT00022245001, GSVIVT0000-9741001) were identified, and represent proteins involved in ubiquitination and subsequent degradation of target proteins. Aspartic proteinase nepenthesin-1 precursor (GSVIVT00032938001) is expressed at higher level in "Nipponbare" in response to phosphorus deficiency [73] and isolated from salt-stress wild rice "*Porteresia coarctata*" [74]. Protein phosphatase 2c (GSVIVT00024072001, GSVIVT00024235001, GSVIVT00001432001) regulates numerous ABA responses [75,76]. Nucleic acid binding proteins (GSVIVT00024387001) control genes expression in response to oxidative stress [77], ABA treatment [78] and abiotic stresses [79]. Exocyst subunit EXO70 family protein H4 (GSVIVT00023109001) has been shown to be involved in the exocytic pathway, which sorts newly synthesized proteins from the endoplasmic reticulum to their final destination at the lysosome, vacuole or plasma membrane [80].

Secondary metabolism

This subcategory contained 4 genes, including a higher level of tropinone reductase (GSVIVT00018424001, +48 fold in INF vs CON) transcript in infected leaves, consistent with previous reports showing it to be more abundant after pathogen infection [81]. Isoflavone reductase-like protein 3 (GSVIVT00019233001, +31 fold in INF vs CON) also has a potential pathogen resistance role because it is involved in biosynthesis of isoflavonoid phytoalexins [82], an important product in resistance to pathogen infection [83,84]. UDP-glucose glucosyltransferase (GSVIVT00002450001, +24 fold in INF vs CON) and galactinol synthase (GSVIVT00019669001, +24 fold in INF vs CON) are reported to be induced by abiotic stresses [85,86].

Cell wall organization

Three genes were classified into this subcategory. Cellulose synthase-like D1 (GSVIVT00014029001, +31 fold in INF vs CON) and beta-expansin 1a precursor (GSVIVT00036225001, +27 fold in INF vs CON) contribute to cell wall synthesis and modification [87,88]. The wound-induced protein (WIN2) (GSVIVT00007452001, +26 fold in INF vs CON) with anti-fungal activity [89] possesses a domain that binds PAMP (pathogen-associated molecular patterns) elicitors (e.g., chitin) [90] and is induced in response to pathogen. In addition, other highly expressed metabolic genes in the INF samples were glucose-1-phosphate adenylyltransferase (GSVIVT00036349001, +24 fold in INF vs CON), cytochrome P450 (GSVIVT00014730001, +70 fold in INF vs CON) and serine acetyltransferase (GSVIVT00007984001, +30 fold in INF vs CON). These transcripts are related to carbohydrate metabolism, photosynthesis and cysteine synthesis. Cysteine synthesis has reported to respond to oxidative stress by calcium signaling [91].

Even though most of these genes have been reported to be biotic or abiotic stresses related, seven high expressed genes in the infected leaves have not been previously reported being associated with stress. They were noted as protein-binding protein (GSVIVT00024408001, +87 fold in INF vs CON), ATPP2-A2 (*Arabidopsis thaliana* phloem protein 2-A2) (GSVIVT00023009001, +56 fold in INF vs CON), putative integral membrane protein (GSVIVT00014704001, +51 fold in INF vs CON), putative phosphate-induced protein (GSVIVT00015203001, +229; GSVIVT00015200001, +37 fold in INF vs CON), ATP-dependent DNA helicase (GSVIVT00016166001, +36 fold in INF vs CON), CW14 (GSVIVT00020834001, +23 fold in INF vs CON), and a hypothetical protein (GSVIVT00017533001, +20 fold in INF vs CON).

Transcripts less abundant in infected leaves

The most striking functions for transcripts less abundant in infected tissue were those associated with

metabolism and defense response to pathogen attack. Fifteen DEGs were detected to be less prevalent in the INF libraries more than 20 fold compared to CON, most of which, such as (-)-germacrene D synthase [92], non-specific lipid transfer protein [93], major histocompatibility complex [94], thioredoxin [95], beta-cyano-alanine synthase [96], expansin [97] and UDP-glucosyltransferase [98] are reported to be positively associated with plant defense responses to pathogen attack. However, our data indicated that the expression level of these transcripts was lower in infected tissues.

Another two transcripts that were less prevalent in infected tissue (GSVIVT00014727001, -35 fold in INF vs CON; GSVIVT00014725001, -41 in INF vs CON) belong to cytochrome P450 family with oxidative function. Interestingly, a novel gene encoding male sterility-related protein was also identified in this group, and its function associated with DM response has not been clarified.

Conclusions

Solexa-based sequencing can be used for analyzing variation in gene expression between two samples. The gene expression level in “Zuoshan-1” leaves infected with PV changed significantly in comparison with control leaves. Analysis of differentially-expressed genes involved in the pathogen infection allows delineation of candidate genes potentially relevant to DM resistance in grapevines.

Methods

Plants material and pathogen infection

One-year-old, certified virus-free seedlings of “Zuoshan-1” were grown and maintained in the greenhouse under a 16-h light/8-h dark photoperiod at 25°C, 85% relative humidity. Control plants were maintained under the same conditions. *P. viticola* was collected from sporulated field leaves and used for the artificial inoculations of surface-sterilized leaves. Infections were conducted by dipping the fourth grapevine leaves in a suspension of 10,000 sporangia per ml pure water. The leaves were covered with plastic bags for one night to ensure high humidity. The fourth unfolded leaf from the shoot apex was harvested from each of three vines, and the three leaves were combined to represent one replicate. Three independent replicates were collected for each sample. Infected leaves were collected every 24 h for 9 days. Control samples were harvested from water-treated leaves incubated under the same conditions.

Preparation of Digital Expression Libraries

Samples from infected leaves from 4 d to 8 d were pooled for RNA isolation and library construction. Comparable control leaves were treated identically and in parallel. Total RNA was isolated from the leaf mixture using a modification of the CTAB method as presented by Murray and Thompson [99]. Sequence tag

preparation was done with the Digital Gene Expression Tag Profiling Kit (Illumina Inc; San Diego, CA, USA) according to the manufacturer's protocol (version 2.1B). Six micrograms of total RNA was extracted and mRNA was purified using biotin-Oligo (dT) magnetic bead adsorption. First- and second-strand cDNA synthesis was performed after the RNA was bound to the beads. While on the beads, double strand cDNA was digested with *NlaIII* endonuclease to produce a bead-bound cDNA fragment containing sequence from the 3'-most CATG to the poly (A)-tail. These 3' cDNA fragments were purified using magnetic bead precipitation and the Illumina adapter 1 (GEX adapter 1) was added to new 5' end. The junction of Illumina adapter 1 and CATG site was recognized by *MmeI*, which is a Type I endonuclease (with separated recognition sites and digestion sites). The enzyme cuts 17 bp downstream of the CATG site, producing 17 bp cDNA sequence tags with adapter 1. After removing 3' fragments with magnetic bead precipitation, the Illumina adapter 2 (GEX adapter 2) was ligated to 3' end of the cDNA tag. These cDNA fragments represented the tag library.

Solexa sequencing

Sequencing was performed by "HuaDa Gene" [100] with the method of sequencing by synthesis. A PCR amplification with 15 cycles using Phusion polymerase (Finnzymes, Espoo, Finland) was performed with primers complementary to the adapter sequences to enrich the samples for the desired fragments. The resulting 85 base strips were purified by 6% TBE PAGE Gel electrophoresis. These strips were then digested, and the single-chain molecules were fixed onto the Solexa Sequencing Chip (flow cell). Each molecule grew into a single-molecule cluster sequencing template through in situ amplification. Four color-labeled nucleotides were added, and sequencing was performed with the method of sequencing by synthesis. Image analysis and basecalling were performed using the Illumina Pipeline, and cDNA sequence tags were revealed after purity filtering. The tags passing initial quality tests were sorted and counted. Each tunnel generates millions of raw reads with sequencing length of 35 bp (target tags plus 3'adaptor). Each molecule in the library represented a single tag derived from a single transcript.

Sequence annotation

"Clean Tags" were obtained by filtering off adaptor-only tags and low-quality tags (containing ambiguous bases). Comparison of the sequences by blastn was carried out using the following databases: NCBI [101], Genoscope Grape Genome database [25] and VBI Microbial Database [26]. All clean tags were annotated based on grape reference genes. For conservative and precise annotation,

only sequences with perfect homology or 1 nt mismatch were considered further. The number of annotated clean tags for each gene was calculated and then normalized to TPM (number of transcripts per million clean tags) [30,102]. Sequences were manually assigned to functional categories based on the analysis of scientific literature.

Identification of differentially expressed genes (DEGs)

A rigorous algorithm to identify differentially expressed genes between two samples was developed [103]. P value was used to test differential transcript accumulation. In the formula below the total clean tag number of the CON library is noted as N1, and total clean tag number of INF library as N2; gene A holds x tags in CON and y tags in INF library. The probability of gene A expressed equally between two samples can be calculated with:

$$P(y | x) = \left(\frac{N_2}{N_1} \right)^y \frac{(x+y)!}{x! y! \left(1 + \frac{N_2}{N_1} \right)^{(x+y+1)}}$$

FDR (False Discovery Rate) was applied to determine the threshold of P Value in multiple tests and analyses [104]. An "FDR < 0.001 and the absolute value of log2-Ratio ≥ 1 " was used as the threshold to judge the significance of gene expression difference.

Real-time RT-PCR analysis

Samples were prepared using the same method mentioned above and total RNA was isolated from the leaf mixture. Experiments were carried out on three independent biological replicates each containing three technical replicates. First-strand cDNA was synthesized from 650 ng DNase (Promega, Madison, Wisconsin, USA) -treated total RNA using "ImProm-II TM Reverse Transcriptase" (Promega, Madison, Wisconsin, USA) and diluted 20 fold as template. Specific primer pairs of twelve randomly selected genes were designed (Table 4) using Primer Express 3.0 and tested by Real-time RT-PCR. Primers specific for *V. vinifera* actin (Forward: AATGTGCCTGCCATGTATGT; Reverse: TCACAC-CATCACCAGAATCC) were used for the normalization of reactions. Experiments were carried out using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) in a StepOne™ Real-Time PCR System (Applied Biosystems). The reaction volume was 20 μ l, including 10 μ l Power SYBR Green PCR master mix, 0.9 μ l 10 mM primer, 2.0 μ l cDNA sample and 6.20 μ l dH2O. The following thermal cycling profile was used: 95°C 10 min; 40 cycles of 95°C for 15 s, 59°C for 1 min; 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. Data were analyzed using StepOne™ Software Version 2.0 (Applied Biosystems). Actin expression was used as an internal

control to normalize all data. The fold change in mRNA expression was estimated using threshold cycles, by the $\Delta\Delta CT$ method [105].

Pathway Enrichment Analysis of DEGs

Pathway enrichment analysis based on KEGG [106] was used to identify significantly enriched metabolic pathways or signal transduction pathways in differentially-expressed genes comparing with the whole genome background. The calculating formula is:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$$

where N is the number of all genes that with KEGG annotation, n is the number of DEGs in N, M is the number of all genes annotated to specific pathways, and m is number of DEGs in M. Q value was used for determining the threshold of P Value in multiple test and analysis [107]. Pathways with Q value < 0.05 are significantly enriched in DEGs.

Additional material

Additional file 1: Complete list of transcripts attributed to *P. viticola*.

Additional file 2: Complete list of involved pathways for upregulated DEGs. Pathways with Q value < 0.05 are significantly enriched for upregulated DEGs.

Additional file 3: Complete list of involved pathways for downregulated DEGs. Pathways with Q value < 0.05 are significantly enriched for downregulated DEGs.

Additional file 4: List of "Zuoshan-1" transcripts upregulated for at least 2 fold in INF library. Two fold and more upregulated genes with pathway annotation in INF library were listed in different categories.

Additional file 5: List of "Zuoshan-1" transcripts downregulated for at least 2 fold in INF library. Two fold and more downregulated genes with pathway annotation in INF library were listed in different categories.

Abbreviations

AFLP: Amplified Fragment Length Polymorphism; BLAST: Basic Local Alignment Search Tool; cDNA: Complementary DNA; CTAB: Hexadecyltrimethylammonium bromide; DEGs: differentially expressed transcripts; NCBI: National Center for Biotechnology Information.

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JW and YLZ carried out the plant material preparation, PV infection, RNA extraction, preparation of digital expression libraries, sequence analysis, and contributed to data interpretation and manuscript writing. HQZ participated in PV infection and RNA extraction. HH contributed to sequence analysis. KMF participated in data interpretation and manuscript modification. JL conceived the study, led the experiment design and coordinated all the research activities, contributed to interpretation of the data, manuscript writing and modification. All authors read and approved the final manuscript.

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