MICROARRAY GENE EXPRESSION ANALYSIS OF GRAPE PLANTS IN RESPONSE TO *XYLELLA FASTIDIOSA* INFECTION

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ABSTRACT
Transcriptional profiling using a custom high-density microarray chip of 20,020 *Vitis* transcripts showed significant variations in responses between the susceptible and resistant genotypes to *Xylella fastidiosa (Xf)* infection. Differentially expressed transcripts reflecting spatial and temporal responses to Pierce's disease (PD) involved in metabolic processes such as diseases resistance, water stress, photosynthesis and cell wall synthesis were identified. The results suggest that *Vitis* responses to *Xf* are genotype and tissue dependent, and are stage specific. VitisExpDB is an online MySQL-php driven relational database that houses annotated EST data. The database will provide genomic resource to grape community for functional analysis for both vinifera and non-vinifera grape varieties and aid in the grape genome annotation.

INTRODUCTION
The impact of Pierce's disease (PD) on the California grape industry has been significant since the introduction and establishment of a more effective vector, *Homalodisca vitripennis*, the glassy-winged sharpshooter (GWSS) (Almeida and Purcell 2003). Development of resistance in grape is stymied by the relatively limited amount of genetic and molecular information regarding genotype specific resistance to PD infection (Davis et al. 1978). From genotypic screening and genetic mapping studies, it was concluded that a dominant allele controls PD resistance and recently, Krivanek et al. (2006) identified a major quantitative trait locus that controls PD resistance and denoted it as 'Pierce's disease resistance 1' (*PdR1*). The above studies confirm that the genetic basis of PD resistance in grapes varies from tolerance to resistance and suggest that host responses to the pathogen are genotype dependent. Further, in the PD resistant genotypes, differential responses between stem and leaf tissues were also noted (Krivanek and Walker, 2005). The results from these studies prompted study of molecular basis of this host / pathogen interaction.

Plants respond to pathogen attack through a variety of signaling pathways consisting of a large number of regulatory as well as effector genes. Microarrays facilitate automated analysis of transcriptional profiling data to enable complete understanding of such gene function and interactions. The goal of this study was to identify and characterize the molecular events in the grape / *Xylella fastidiosa* (*Xf*) interaction using genome wide transcriptome profiling between resistant and susceptible genotypes and among the different tissue types.

OBJECTIVES
1. Microarray gene expression analysis.
2. Develop of a grape transcriptional relational database.

RESULTS
Objective 1 - Microarray gene expression analysis.
***Custom microarray chip design*:** Previously, we have characterized transcriptomes (5,794 ESTs) from 12 tissue specific (stem, leaf and shoot) subtractive suppression hybridization (SSH) libraries. All the sequenced ESTs that are at least 100 bp in length (5421 ESTs) were submitted to the NCBI's ESTdb under the accession numbers DN942225 to DN947645. These ESTs and all the other EST sequences publicly available till July of 2005 were analyzed to deduce a non-redundant set of 20,020 ESTs with 1,947 from the SSH libraries, including 40 from the cDNA-AFLP experiments, 10,014 from *V. vinifera*, 5,470 from *V. shuttleworthii*, 1,219 from *V. aestivalis*, 780 from *V. rupestris* x V. sp and 588 from *V. riparia.* Nine individual 60-mer probes were designed for each EST. A total of 191,450 probes were selected for the entire set and there were two spots for each probe on the slide totaling 382,900 spots per slide.

Experimental set-up: Total RNA from stem and leaf tissues was hybridized to 36 slides (eighteen for each explant) in a two-color experiment using the monochromatic dyes Cy5 and Cy3. RNA from three time points: early (1 week), mid (6 weeks) and late (10 weeks) stages of disease development from both infected and non-infected tissues of resistant and susceptible genotypes was analyzed. For each time point, there were three slides (biological replicates) including a dye flip.

Data analysis: For each gene and for each explant (stem and leaf) there were 54 data points per each stage (18 per slide x 3 biological replications) of disease development. Data representing raw spot intensities generated by the GenePix software were analyzed using the SAM microarray analysis software to generate fold differences and q-values. Clustering of the significantly differentially expressed genes was carried out using TMEV software.

Overview of transcriptional responses: A total of 8926 transcripts (5,299 individual ESTs and the rest are overlaps) showed statistically significant differentially regulation in the above experiments, with nearly 30 % of those being cloned from the SSH libraries (Table 1). Out of 5,299 individual transcripts that were responsive, 58.65 % (3,108 ESTs) were specific to a certain stage. Below we briefly describe the expression pattern of the major categories of these differentially expressed genes.

Table 1. Microarray hybridization identified differential expression in grape stem (**A**) and Leaf (**B**) tissues in response to *Xf* infection in both susceptible and resistant genotypes. Microarray analysis was carried out using the SAM (Significance of Microarray Analysis) software, with a q-value cutoff of 0.5 %. Values are presented for genes that are at least two-fold regulated.

(A) Stem tissue

Stage	Response	Genotype			
		9621-67		9621-94	
		# Of ESTs	Fold-Change	# Of ESTs	Fold-Change
Week-1	Up-regulated	294	2.0 - 6.44	9	2.0 - 2.92
	Down-regulated	421	0.49 - 0.09	2	0.48, 0.4
Week-6	Up-regulated	230	2.0 -5.05	938	2.0 – 38.9
	Down-regulated	55	0.49 - 0.22	665	0.49 - 0.025
Week-10	Up-regulated	451	2.0 -18.16	459	2.0 – 37.26
	Down-regulated	291	0.49 - 0.14	995	0.49 - 0.03

(B) Leaf tissue

Stage	Response	Genotype			
		9621-67		9621-94	
		# Of ESTs	Fold-Change	# Of ESTs	Fold-Change
Week-1	Up-regulated	269	2.0 – 7.47	0	-
	Down-regulated	43	0.49 - 0.28	6	0.48-0.36
Week-6	Up-regulated	82	2.0 -5.68	151	2.0 – 14.7
	Down-regulated	18	0.49 - 0.33	37	0.49 - 0.23
Week-10	Up-regulated	328	2.0 -15.63	1363	2.0 - 53.02
	Down-regulated	590	0.49 - 0.05	1229	0.49 - 0.04

1. Disease related proteins

Selective induction of 19 transcripts known to be associated with defense responses was observed in stem tissue of the resistant genotype with 2 to 6.5 fold upregulation. This includes transcripts such as MAP kinase, transcription factor EREBP1, Disease resistance protein ADR1, mannitol dehydrogenase among others. Similarly, in the leaf tissue, several defense related transcripts were differentially upregulated with 2 to 3.4 fold (PR1, PRB1-3, ABC transporter-like protein). In the susceptible genotype, only the leaf tissue showed selective induction of defense related transcripts. A large number of transcripts belonging to serine/threonine kinasePR5K, along with others were several fold induced. Transcripts such as defense-related protein, Germin-like protein subfamily 3, endochitinase B precursor were expressed in both the genotypes and could indicate broad genotype independent response.

2. Photosynthesis

Expression levels of leaf tissue transcripts involved in photosynthesis from the susceptible genotype showed a clear indication of down regulation (Figure 1A). Some of the down regulated transcripts included RUBISCO (0.303-fold) Photosystem I reaction center subunit III, chloroplast precursor (0.31-fold) and Chlorophyll a-b binding protein AB80 (0.26-fold). On the other hand, expression of the DREbinding ERF3 was upregulated (4.26-fold). This suggests an increased stress in the susceptible genotype compared to the resistant genotype at this stage of disease development.

3. Water stress proteins

In the leaf tissue of the susceptible genotype, 10 weeks after infection, expression of several of the drought and water stress associated transcripts was upregulated such as Betaine aldehyde dehydrogenase (6.23-fold), heat shock transcription factor 1 (2.5-fold) and dehydrin (3.55) (Figure 1B). Expression of these transcripts was unchanged in the resistant genotype at the same stage of disease development. On the other hand, some of the transcripts in the leaf tissue showed several fold up regulation in the susceptible genotype compared to the resistant genotype, such as galactinol synthase a protein induced by water stress (15.1-fold and 38.4-fold in the 9621-67 and 9621-94 genotypes respectively) and the cold and drought regulated protein (CORA; 2.4-fold and 9.77-fold respectively). In the stem tissue from the same genotype, up regulation of transcripts belonging to osmotic stress-activated protein kinase (6.49-fold), neoxanthin cleavage enzyme (8.32-fold) and DT-regulated protein (3.66) was observed.

4. Cell wall and xylem proteins

Genes involved in cell wall degradation such as pectinesterases, exopolygalactruonase and other senescence associated proteins such as caffeoyl-CoA O-methyltransferase were several fold upregulated in the susceptible 9621-94 genotype compared to the resistant 9621-67 genotype. Expression of cellulases such as endoglucanase and that of xyloglucan endotransglucosylase was several folds higher in the 9621-94 genotype (0.3-fold and 37.95-fold respectively in the resistant 9621-67 and susceptible 9621-94 genotypes). Similarly, down regulation of ESTs such as Germin like proteins and monocopper oxidase precursor in the 9621-94 genotype was more pronounced than in the 9621-67 genotype. Expression of glucanases was downregulated in both the genotypes after 10 weeks of infection.

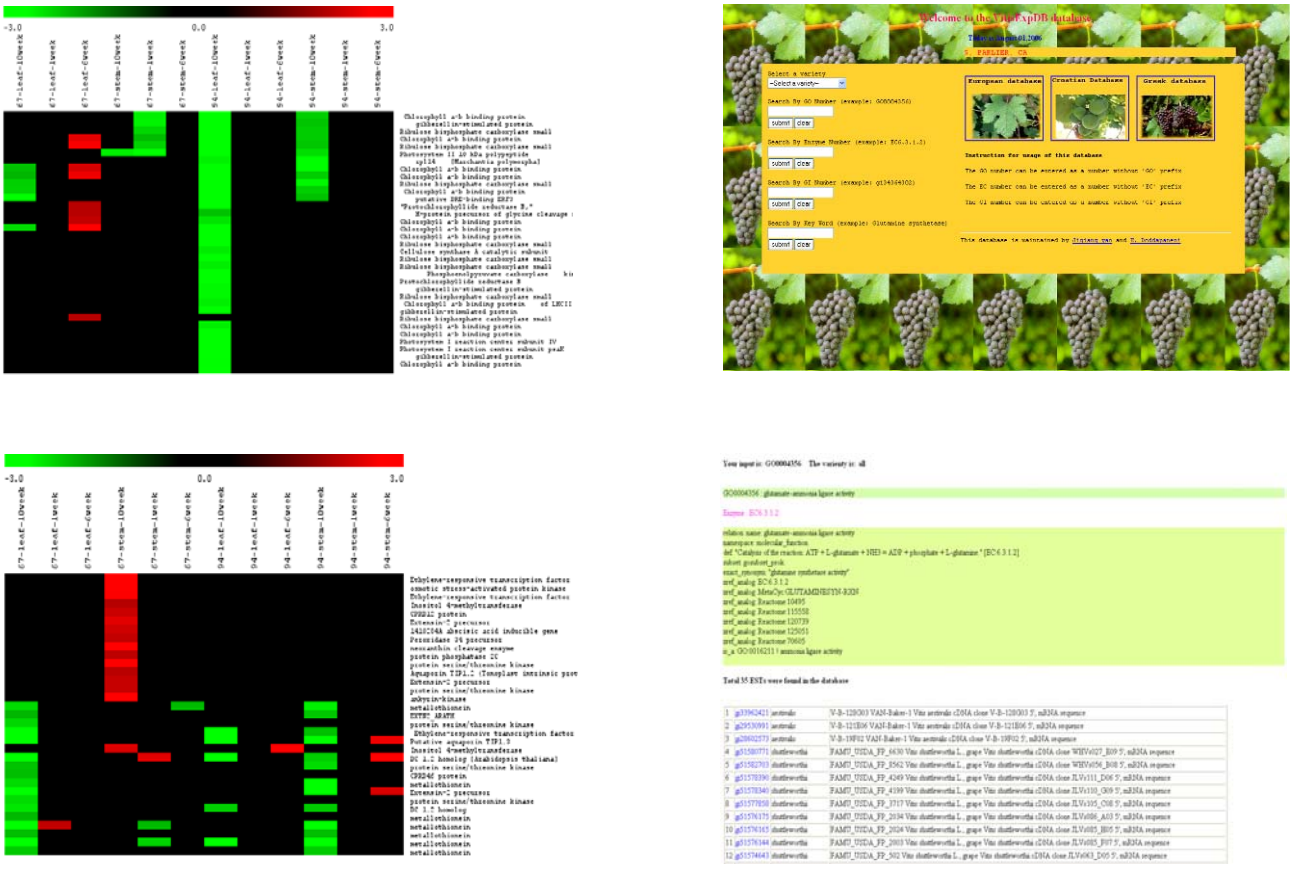

Figure 1. Expression profiling of the differentially regulated transcripts. Red indicates transcriptional activation and green represents repression. Transcripts that are not significantly regulated are shown in black. **(A)** Photosynthesis related transcripts. **(B)** Water-stress related transcripts. Hierarchical clustering was performed using TMEV. The results show a clear segregation of the genotype and tissue types in response to pathogen infection. **(C & D)** VitisExpDB is an online MySQL-php driven relational database that houses annotated EST and gene expression data for both vinifera and non-vinifera grape varieties.

Objective 2 - Develop of a grape transcriptional relational database:

VitisExpDB is an online MySQL-php driven relational database that houses annotated EST data for both vinifera and non-vinifera grape varieties. The database includes all the EST data in the public domain from both vinifera and non-vinifera varieties. In the present study, using the latest Gene Ontology (GO) terminology, a uniform structural vocabulary was

developed for the above grape varieties. Further, extensive cross referencing is allowed to retrieve the data using multiple search indices. Future plans include expansion of the database to incorporate all the microarray expression data from our as well as other reported studies. ESTs of *V. vinifera* and *non-vinifera* grapes (*Vitis vinifera*, *V. shuttleworthii*, *Vitis hybrid cultivar*, *V. rupestris* x *V. arizonica*, *V. aestivalis*, *V. riparia*, *V. pseudoreticulata*, *V. cinerea* x *V. rupestris*, *V. cinerea* x *V. riparia* are currently included.

Database architecture and Web interface: The relational database is powered by an Apache 2.0 server and was developed using MySQL 5.0 as the database management system on Red Hat Enterprise Linux 4 RPM (x86). EST sequence sets were downloaded from NCBI GenBank (UniGene, dbEST) and annotated with GO terms Sequence similarity search was carried out using the default blast parameters and a cut off E value of 10-4. On the main search page, a drop down menu that lists the variety (s) to be queried is provided. The database can be searched by Gene Ontology ID, GenBank ID, enzyme number, or by inputting key word (s) (Figure 1C). The result page displays the number of ESTs matching the query, individual EST sequence, its description, EC number, and its Gene Ontology classification (Figure 1D). VitisExpDB database is available at http://cropdisease.ars.usda.gov/~fruit_tree/.

CONCLUSIONS

Characterizing the molecular basis of the grape response to *Xf* is critical to understanding the mechanisms of PD resistance and pathogenesis. Based on our transcript profiling, it is clear that grape plant response to *Xf* infection is different among tissues between resistant and susceptible genotypes, and early and late stages. While a broad spectrum and presumably non specific plant response was observed for defense and water stress related protein expression in the susceptible genotype, a majority of this did not overlap with the resistance genotype response. Further, transcript profile also indicated a higher level of water deficiency in the susceptible genotype compared to resistant genotype.

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COMPARATIVE PROTEOMIC ANALYSIS OF STEM TISSUE AND XYLEM SAP FROM PIERCE'S DISEASE RESISTANT AND SUSCEPTIBLE GRAPEVINES

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ABSTRACT

Analyses of host plant resistance / susceptibility mechanisms to *Xylella fastidiosa* (*Xf*) infection are critical for understanding host-pathogen interactions. Proteomic analyses of stem tissue and xylem sap samples were initiated to complement genomic approaches employed in elucidating Pierce's disease resistance mechanisms. Samples from one highly resistant and two susceptible grape genotypes were collected at multiple time points post-inoculation from control and *Xf*-inoculated plants. Two-dimensional gel electrophoresis revealed numerous proteins that were differentially expressed and dependent on plant genotype and/or inoculation treatment. Proteins identified by oMALDI-TOF comprised a wide range of functional types. The importance of these proteins with respect to host-pathogen interactions will be investigated further.

INTRODUCTION

While numerous factors including temperature, fertilization and time are known to affect xylem sap chemistry (Andersen and Brodbeck, 1989a, 1989b, 1991; Andersen et al., 1995, 2004b), the protein composition of grape xylem sap in response to *Xylella fastidiosa (Xf)* infection has not been investigated in detail to date. In other host plant-pathogen systems, xylem sap proteins were shown to be important in suppression of disease development (Ceccardi et al., 1998; Guo et al., 1993; Nemec, 1995; Reimers and Leach, 1991; Reimers et al., 1992; Rep et al., 2002; Young et al., 1995). Without a doubt, due to its xylem limited growth habit, *Xf* 's growth and development are influenced by xylem sap characteristics. Thus manipulation of the xylem sap composition presents a promising venue to interfere with *Xf* infections.

Disease expression in stems of grape vines results in blocking of water flow to the shoot and, thus, is critical to the lethal nature of Pierce's disease (PD). *Xf* infection results in uneven cane maturation which expresses itself in the formation of green-islands. The irregular nature of green-islands suggests the involvement of localized rather than systemic signals in the formation of the observed spatial symptomology. The importance of *Xf* populations in stems in respect to PD resistance of grape genotypes was also illustrated in recent studies (Krivanek et al., 2005; Krivanek and Walker, 2005). Thus, examination of stem tissue provides an opportunity to identify important aspects of plant-pathogen interactions.

Examination of xylem sap and stem protein makeup is a new approach that allows us to complement our genomic studies conducted on the same susceptible and resistant sibling genotypes employed in this study.

OBJECTIVES

1. Discover xylem sap and stem proteins differentially expressed in PD resistant and susceptible grapes in response to *Xf* infection.
2. Identify differentially expressed proteins from xylem sap and stem induced by *Xf*.

RESULTS

Objective 1. Discovery of differentially expressed proteins.

PD resistant (9621-67) and PD susceptible (9621-94) genetic lines selected from a segregating population of *V. rupestris x V. arizonica* as well as *vinifera* grape, Chardonnay were used in this comparative study. We completed expression experiment conducted in the greenhouse where treatment and control grapevines were mechanically inoculated with *Xf* suspension respectively. Leaf and stem tissues were then collected at ten time points from as early as day one post inoculation up to three months when PD symptom was fully expressed in susceptible grapes. A separate set of grapes (same genotypes and treatments as above) was grown in the greenhouse for xylem sap protein extraction at 2 time points post inoculation. The xylem sap was extracted using a pressure chamber following the same sampling scheme as above. Samples (stem, leaf and