FUNCTIONAL GENOMICS OF THE GRAPE-*XYLELLA* INTERACTION: TOWARDS THE IDENTIFICATION OF HOST RESISTANCE DETERMINANTS

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ABSTRACT

In silico mining of EST data, Real Time PCR, and Affymetrix GeneChip technology was used to characterize the transcriptional response of *Vitis vinifera* to the Pierce's disease (PD) pathogen *Xylella fastidiosa (Xf)*. We have determined that susceptible *V. vinifera* responds to *Xylella* infection with a massive re-direction of gene transcription. This transcriptional response includes the up regulation of transcripts for phenlypropanoid and flavonoid biosynthesis, ethylene production, adaptation to oxidative stress, and homologs of pathogenesis related (PR) proteins. In addition to highlighting potential metabolic and biochemical changes that are correlated with disease, the results suggest that susceptible genotypes respond to *Xylella* infection by induction of limited defense response.

A long-standing hypothesis states that PD results from pathogen-induced drought stress, with the consequent development of disease symptoms. To test this hypothesis, we compared the transcriptional and physiological response of plants treated by pathogen infection, low or moderate water deficit, or a combination of pathogen infection and water deficit. We determined that the transcriptional response of plants to *Xylella* infection is not the same as the response of healthy plants to moderate water stress. However, there is an apparent synergistic interaction between water stress and disease, such that water stressed plants exhibit a stronger physiological and transcriptional response to the pathogen. Qualitative and quantitative estimates of gene expression derived from the Affymetrix gene chip were confirmed by a combination of Real Time PCR and *in situ* hybridization analysis with ~20 candidate marker genes.

Real Time PCR analysis involving six marker genes was used to survey the specificity of *Xylella*-induced gene expression under field conditions. The results demonstrate that the marker genes are up-regulated in response to *Xylella* infection but not in response to the other pathogens assayed, including common viral, nematode and fungal pathogens, or by *Phylloxera* infestation or herbicide damage. Similarly, moderate drought stress did not result in increased transcript levels for these marker genes. By contrast, each of the marker genes was strongly induced in non-infected leaves where the vascular system was compromised by biotic or abiotic factors, including girdling by insect damage and severe drought stress leading to death. We hypothesize that an aspect of xylem dysfunction, but not drought stress per se, is one trigger for *Xylella*-induced gene expression.

INTRODUCTION

All organisms adapt to external stressors by activating the expression of genes that confer adaptation to the particular stress. In the case of Pierce's disease (PD), such genes are likely to include those coding for resistance or susceptibility to *Xylella fastidiosa (Xf)*.

Genomics technology offers an opportunity to monitor gene expression changes on a massive scale (so-called "transcriptional profiling"), with the parallel analysis of thousands of host genes conducted in a single experiment. In the case of PD of grapes, the resulting data can reveal aspects of the host response that are inaccessible by other experimental strategies. In May of 2004, the first Affymetrix gene chip was made available for public use, with ~15,700 *Vitis* genes represented. This gene chip has been developed based primarily on a collaboration between the Cook laboratory and researchers at the University of Nevada-Reno (Goes da Silva et al., 2005). With the arrival of the Affymetrix gene chip, we are poised to make a quantum leap in the identification of host gene expression in response to *Xf*.

In addition to enumerating differences between susceptible and resistant genotypes of *Vitis*, this research is testing a long-standing but largely untested hypothesis that pathogen-induced drought stress is one of the fundamental triggers of PD symptom development. The utility of this type of data will be to inform the PD research community about the genes and corresponding protein products that are produced in susceptible, tolerant and resistant interactions. Differences in the transcriptional profiles between these situations are expected to include host resistance and susceptibility genes, and thus

provide the basis for new lines of experimental inquiry focused on testing the efficacy of specific host genes for PD resistance. It should be possible, for example, to determine the extent to which resistance responses in grapes are related to well-characterized defense responses in other plant species (e.g., Maleck et al., 2002; Tao et al., 2003; de Torres et al., 2003).

Three co-lateral benefits from the identification of pathogen-induced genes are: (1) the promoters for such genes are candidates to control the expression of transgenes for resistance to PD, (2) the protein products of induced genes may have roles in disease resistance, and (3) knowledge of host gene expression can be used to develop improved diagnostic assays for disease. In a related project, we are currently characterizing pathogen-responsive promoters, which will facilitate testing of candidate genes for resistance phenotypes.

OBJECTIVES

1. Identify genes and gene pathways in susceptible *V. vinifera* correlated with *Xf* infection: (a) identify *Xylella*-responsive genes in *V. vinifera*, (b) distinguish early from late gene expression, and (c) determine the correlation between drought stress and PD.
2. Determine host genotype affects on gene expression in response to *Xylella* infection: (a) susceptible *V. vinifera* compared to resistant genotypes of *Vitis* and *Muscadinia* species, and (b) comparison of pathogen-induced gene expression with gene expression triggered by salicylic acid and ethylene.
3. Detailed analysis of candidate genes: **(**a) Real Time PCR to validate candidate genes identified in objectives 1 and 2, (b) Real Time PCR to study kinetics and specificity of the host response in susceptible and resistant genotypes, and (c) *in situ* hybridization to establish precise location of plant gene expression relative to bacterial infection.

RESULTS

Testing the effect of plant water status on PD

Two lines of evidence suggest that plant water status may have a significant impact on the development of PD symptoms. First, it is frequently observed that well-watered plants develop reduced symptoms relative to water-stressed plants. Thus, one might expect to see an enhanced transcriptional response in plants that are both water-stressed and infected by the pathogen. Second, it has been proposed that *Xylella* infection of xylem elements obstructs water flow, leading to whole-plant water stress and consequently to symptom development. Despite the logic of this reasoning, a causal relationship between *Xylella* infection and water stress has not been established. It is noteworthy, that the "water stress" hypothesis does not explain the absence of symptoms early in the season, even though high pathogen titers can be observed at this phase of disease, and it does not explain the absence of symptoms in tolerant genotypes of grapes, which can be heavily infected by the pathogen but without disease.

The experimental design described below permits a comparison of (1) pre-symptomatic and post-symptomatic host responses, (2) drought stressed versus diseased individuals, and (3) the interaction between drought stress and pathogen infection. In total, fourteen different transcriptional states that were compared to address these issues.

The experimental design involved 42 three-year-old vines of Cabernet Sauvignon clone 8 grafted to Freedom rootstock. In the spring of 2004, potted vines were moved from greenhouse to growth chamber prior to budbreak. Subsequent to a 3 to 4 week acclimation period, vines were pruned to produce a uniform shoot architecture consisting of two shoots per plant and ten leaves per shoot. Plants were grown in a block design of 3 rows with all treatments randomized in each row. Water use was calculated by watering 5 plants to field capacity and using a mini-lysimeter to establish water usage over a 24-hour period. The resulting average value was used to define 100% estimated water use. Plants were watered either at 100% water usage, 50% water usage (mild stress), or 25% water usage (moderate stress) throughout the remainder of the experiment. For each plant, measurements were made on the second leaf opposite to cluster to infer the level of drought stress pre- and post-veraison. Gas exchange and stomatal conductance values were obtained with a Licor 6400 gas exchange analyzer. C^{13}:C^{12} ratios were measured on the same leaf samples used for transcriptional profiling to estimate long term effects of treatments on stomatal conductance, gas exchange and water use efficiency. On April 12, corresponding to full bloom, plants were either inoculated with a suspension of *Xf* or mock inoculated with water. Four weeks following inoculation, the third and fourth leaves were harvested from three plants of each treatment type. At 8 weeks following inoculation, when symptoms were evident on infected individuals, the remaining plants (3 from each treatment) were harvested. On the day of harvest for arrays the 5th leaf from each plant was destructively sampled to measure "pre-dawn" water potential. Symptom development was recorded using a visual scale.

RNA was extracted from tissue using protocols that we have optimized for quality and yield of RNA from grape (Iandolino et al., 2004). cRNA synthesis was carried out according to procedures described in the Affymetrix technical manual. Hybridization and data collection were performed using standard Affymetrix protocols, with the aid of the University of California, Davis microarray facility in the University of California, Davis Genome Center. Technical and biological replicates demonstrated highly consistent results within and between similarly treated samples. Quality control analyses were conducted using GCOS 1.2 (Affymetrix), Dchip (Li and Wong, 2001), and the Affy R package. Robust Multichip Average or RMA (Irizarre et al., 2003) was used to estimate differentially expressed genes by two different strategies: a) application of t-test and fold change filters (Sottosanto et al., 2004); and b) false discovery rate determinations using Significance

Analysis of Microarray (SAM) Data (Aubert et al., 2004). Differential regulation was assessed by comparison to uninoculated control plants grown under identical conditions.

In total, 238 genes were identified as being differentially expressed (T-test a < 0.05; ≥2-fold induction) in response to *Xf* treatment or drought stress (Figure 1). There are 2 primary conclusions from this study: First, we have identified several genes where expression is induced strongly in diseased tissue and where drought stress does not appear to impact this transcriptional response. The majority of such genes have predicted roles in defense and cell wall metabolism. Second, a large fraction of the *Xylella*-induced transcriptome is synergistically modified in plants that are doubly-treated by pathogen infection and moderate drought stress. These genes fall into two categories: synergistically upregulated are primarly from the flavonoid biosynthesis pathway, while synergistically down regulated are primarily from the photosynthesis pathway. These results are consistent with the existence of two distinct classes of transcriptional response in grapes to *Xylella*. One response is sensitive to plant water status and results in redirection of flavonoid synthesis and photosynthesis genes, and one response is independent of plant water status leading to the activation of defense-related transcripts. Although we observed limited overlap in the genes induced in response to moderate drought stress and the genes induced in diseased tissue, we cannot rule out the possibility that a more severe drought stress may lead to an increase in the coincidence of PD and drought-associated gene expression.

As shown in Table 1, physiological measurements of the plants used for microarray analysis also suggest an additive interaction between water stress and PD. We note that the level of water stress imposed in these experiments induced an acclimation response in treated plants, as evidenced by measurements of stomatal conductance, internal CO_2 concentrations and transpiration rates. However, reductions to pre-dawn water potential and net assimilation rates document a clear water stress response. By contrast, pathogen infection had a strong influence on virtually all of these parameters. Moreover, drought stress combined with pathogen infection tended to increase the magnitude of change in all parameters assayed. These results suggest a reduced capacity for acclimation to water stress in infected plants and they agree well with the results of gene expression, described below.

A 2-Dimensional hierarchical cluster generated with the DChip software (Li and Wong, 2001) was used to depict the expression 238 genes that were responsive to one or mor of the treatments. The most striking aspect of this particular analysis is the massive transcriptional response that occurs in infected and symptomatic plants. Major categories and/or expression patterns of genes identified so far are described briefly below.

I. Disease related gene expression.
Seventeen transcripts were annotated as disease related genes, including many pathogenesis related or PR protein genes. On average these genes were up regulated 7-fold in response to pathogen infection. Expression of these genes was not influenced by drought either in healthy or diseased plants. The sole exception are two PR protein genes that were down regulated 2.5-fold in response to drought stress, but up regulated >10-fold in response to the pathogen. These results suggest the occurrence of a pathogen-specific defense response in susceptible *V. vinifera*.

II. Photosynthetic gene expression.
One of the most common responses of plants to drought stress is a down regulation of photosynthesis. Consistent with physiological measurements, 11 photosynthesis-related transcripts were significantly down regulated in *Xylella*-infected plants. While moderate water stress had little or no effect on expression of these genes, the combination of pathogen infection and water stress resulted in an even greater reduction in gene expression compared with either treatment alone. *Xylella* causes a decrease in photosynthetic gene expression that is accentuated by reduced water availability.

III. Flavonoid pathway gene expression.
The largest transcriptional effect of *Xylella* infection was a massive re-direction of enzymes and regulatory proteins for flavonoid biosynthesis. In total, 27 genes were 4-fold upregulated in *Xylella* infected plants, compared to healthy control plants. Approximately 50% of these transcripts were induced an additional 2.5-fold when drought stress and *Xylella* infection were combined. The transcription of flavonoid pathway genes was not significantly affected by drought stress alone. *Xylella* causes an increase in flavonoid gene expression that is accentuated by reduced water availability.

IV. Genes induced uniquely in the interaction between disease and drought.
Twelve genes were unaffected by either drought or *Xylella* infection, but were significantly induced in plants that were challenged with both *Xylella* and water stress simultaneously. On average, these genes were induced 3.5-fold in double-treated plants. Annotations for these genes do not suggest function in a common pathway.

V. Osmotic stress and cell wall modifying enzymes.

Eleven *Xylella*-associated transcripts have predicted roles in cell wall metabolism (e.g., expansins, enzymes involved in pectin degradation and pectin modification) or osmotic stress (e.g., galactinol synthase, dehydrin proteins and several aquaporins). These genes were induced an average of 5-fold in *Xylella* infected tissues. None of these 11 genes were up-regulated in response to water stress alone, and only the dehydrin and galactionol synthase genes showed evidence of synergy between *Xylella* and drought stress. Cell wall modification genes (expansins, pectin esterases, pectatelyases, polygalacturonases, etc.) were among the major class of water stress repressed genes in *Arabidopsis* (Bray, 2004). In the current study, these genes were induced by the pathogen, providing a possible counterpoint to the argument in favor of *Xylella*-induced drought stress.

CONCLUSIONS

In summary, a wide array of genes are up regulated (or in some cases down regulated) in grapes in response to *Xylella* infection. We found limited correlation between the nature of genes induced by moderate drought stress and the genes induced by pathogen infection. Interestingly, however, the results suggest a synergistic effect of drought stress on *Xylella*-induced gene expression. We have also identified numerous genes where induction was specific to the pathogen, and not synergistic with drought. This later class of genes included pathogenesis related protein genes and genes involved in plant cell wall metabolism. Ongoing experiments are using Real Time PCR to validate and extend the Affymetrix GeneChip results (data not shown) and to determine the spatial pattern of gene expression for the various classes of transcriptional response, as shown by example for gene 8946 in Figure 2.

Our earlier work with a small set of pathogen-induced genes has permitted us to characterize the kinetics and specificity of the host response to *Xylella*, and to isolate and begin the characterization of *Xylella*-reponsive gene promoters. The recent results, reported above, provide a large suite of new genes and predicted biochemical pathways for investigation. We suggest that these results are a first step toward a comprehensive understanding of host responses to PD, and the relationship of disease to whole plant physiology including water relations, photosynthesis and defense responses. Our continuing work will explore in detail the relationship between gene expression in resistant and susceptible plants, and to begin more precise analysis of the spatial relationship between gene expression and pathogen localization. Moreover, we anticipate providing many additional and potentially useful gene promoters to Dave Gilchirst's project to develop a pathogen-inducible transgene system. How will these technologies help in solving PD? In the short term they will:

1. Provide gene-promoters for effective genetic engineering in grapes.
2. Inform us about the nature of host responses to *Xylella* infection.
3. Allow pathogen detection based on Real Time PCR using a "biomarker" strategy.
4. *In the long term*, transcriptional profiling will identify candidate genes and gene pathways that may confer resistance to the pathogen (*Xf*).

Other strategies, such as reverse genetics and analysis of natural genetic variation, will be needed to establish a causal role for candidate genes.

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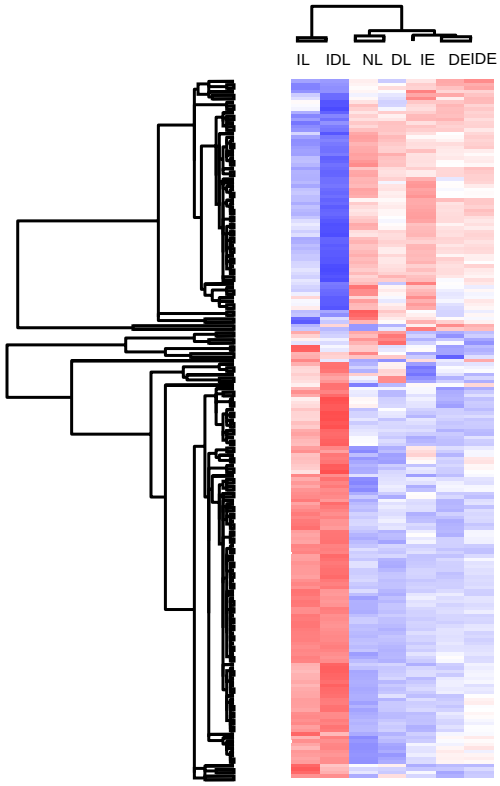

Table 1. Summary of physiological measurements for water relations and photosynthesis**.**

		Physiological parameter[b]				
Treatment	plants	Y_p	$A_{n,max}$	g_s	C_i	E
Non-inoculated	6	0.27	25.11	0.2612	1239.17	4.34
Mock-inoculated	6	0.317	25.75	0.2211	1188.34	3.79
Xf-inoculated	8	0.49	16.22	0.0721	817.00	1.50
Mild stress	7	0.434	19.98	0.2196	1245.00	3.78
Double treatment	8	0.583	12.14	0.0261	556.5	0.64

[b]Physiological parameters were measured 8 weeks after the treatment. Yp: pre-dawn water potential (-MPa), $A_{n,max}$: net assimilation (mmol $CO_2/m^2/s$) (measured at saturating CO2 and light), g_s: Stomatal conductance (umol $H_2O/m^2/s$), Ci: Internal CO_2 concentration (mmol CO_2/mol air), E: transpiration rate (mmol $H_2O/m^2/s$).

Figure 1. 2-Dimensional hierarchical cluster analysis of 24 microarrays from the moderate drought stress condition. 238 transcripts were identified with a minimum of 2-fold induction and a T-test score of a=0.05. Red = increased expression; Blue = decreased expression; White = no change in expression. I=infection; D=drought; N=healthy; E= prior to symptom development; L=subsequent to symptom development.

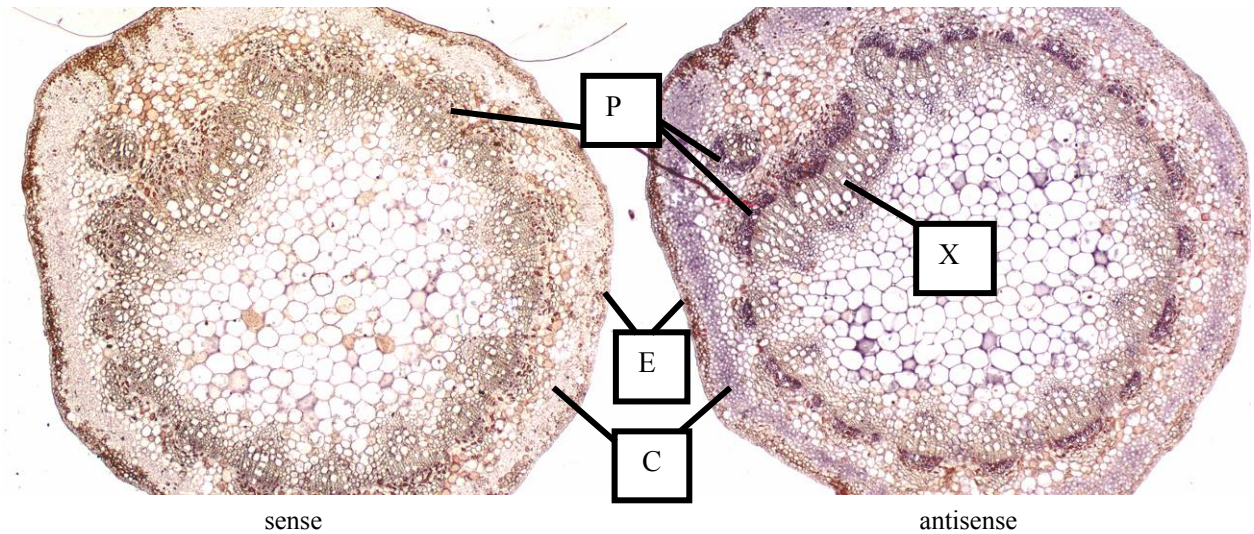

Figure 2. *In situ* localization of candidate gene 8946. Note intense staining in phloem and xylem associated pyrenchyma, indicating *Xylella*-induced gene expression in living tissue adjacent to differentiated xylem.