AVF Annual Progress Report 2002 (April – January)

I. Project Title: Enhancement of Stress Tolerance in *Vitis vinifera*.

II. Principal Investigators:

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Jennifer Weller and Chunhong Mao, Virginia Bioinformatics Institute, 1800 Kraft Drive, Suite 1100 Corporate Research Center - Virginia Tech, Blacksburg, VA 24061 (they were not included in the original proposal, but see below).

III. Summary: (500 words or less)

Abiotic stresses affect important aroma, flavor and color components by altering metabolite composition, improving wine quality and human health benefits. Regulated deficit irrigation has been used successfully to grow grapes with less water, an important feature in arid regions such as Nevada. As a first step toward understanding how growth is affected and wine quality improvements might arise following abiotic stress exposure, we have initiated an expressed sequence tag (EST)-based gene discovery program focused solely on stressed plants. We constructed cDNA libraries from mRNA isolated from leaf, berry and root tissues of *Vitis vinifera* cv. Chardonnay, exposed to various abiotic stress conditions. To date, we have met our goals for the last two years and sequenced more than 20,000 ESTs, approximately 6,700 each from the leaf and berry libraries and 9,500 from the root libraries. Raw sequence data were processed through an automated EST analysis pipeline (ESTAP) developed at the Virginia Bioinformatics Institute (VBI; Blacksburg, VA) in collaboration with UNR and S.R. Noble Foundation (Ardmore, OK). Initial sequence analysis revealed approximately 50% novel or functionally unknown genes and a low redundancy of transcripts (38%). All EST data generated to date from leaf and berry libraries have been deposited in GenBank and are freely available to the public at the NCBI dbEST and ESTAP websites. Data from root libraries is currently being subjected to rigorous cleansing to avoid deposition of EST data from contaminating soil microorganisms.

IV. Objectives and Experiments Conducted to Meet Stated Objectives:

1) **To enhance drought, salinity and cold tolerance in grapevine by the over-expression of *Arabidopsis CBF/DRE*s and *Vitis vinifera CBF/DRE* orthologs.**
2) **To develop cDNA libraries from drought, salinity, and cold stressed leaves and fruits of *V. vinifera*.**
3) **To discover other genetic tools for engineering improved stress tolerance.**
4) **To conduct comparative metabolite profiling in grapes from well-watered and water-deficit treated vines and work towards determining the genetic basis of the factors responsible for improving the quality of wine produced from drought-stressed plants.**

The objectives of the original 3-year research proposal are listed above. We concentrated on objective 2 as advised by last year's proposal reviewers.

Development of cDNA libraries and ESTs: In the first year, we exposed plants to a variety of abiotic stresses (drought, cold, heat, salt, and flooding; see Table 1 for details) to develop mixed abiotic stress cDNA libraries.

Table 1. Abiotic stress treatments applied to Chardonnay plants used to make cDNA libraries. The water potential of the leaves was measured with a pressure chamber at midday as described (McCutchan and Shackel 1992). Berries were harvested at 7 different developmental stages with maturity measured by the Brix/Titratable Acidity ratio.

Treatment	**Organ harvested**
Salt: (20 mM Na_2SO_4, 40 mM NaCl, 10 mM $CaSO_4$) for 2, 24 and 144 hours; note these Cl^- concentrations would be lethal with long-term exposures	Root and Leaf
Drought: potted plants not watered (leaf water potentials ranged from –1.4 to –2.2 MPa)	Root and Leaf
Drought: field plants harvested at 7 different berry developmental stages (preveraison to over-ripeness) with water potentials ranging from –1.0 to –1.7 MPa	Leaf and Berries
Cold: 2 and 24 h at 4°C; incremental decrease in night temperature (2°C per night) to 4°C over 6 days; 6 days of 4°C nights	Root and Leaf
Heat: 20 min at 42°C; incremental rise (2°C per day) to 42°C over 6 days	Root and Leaf
Flooding: Roots (of potted plants) under water for 24h	Root and Leaf

Leaves and fruit were obtained from these plants, but not roots, since it was not feasible to apply other stresses to field grown plants. Field grown plants were exposed to drought stress. Additional stresses were applied to greenhouse-grown potted plants that were less than a year old. Leaves and roots could be collected from these plants, but because of their age, no fruit could be collected. To increase the diversity of transcripts we applied both acute and chronic forms of stress and extracted RNA at different time periods. Chronic levels would allow transcripts involved in long-term acclimation to be expressed and characterized. Since short-term, high-salt stress would mimic the short-term, acute drought stress, we only applied a long-term chronic level of salt stress. Both Cl^- and SO_4^{-2} salts were added to increase diversity and more realistically simulate soil conditions. Temperature stresses were applied in a growth chamber. Tissue was collected from both acute and chronic stress treatments to capture the full repertoire of genes.

Leaf, root and berry RNA was extracted from stressed plant organs using a modified protocol for RNA extraction of spruce (Wang et al. 2000). Libraries were made using the Uni-ZAP-XR cDNA synthesis kit (Stratagene, Inc.).

V. <u>Summary of Major Research Accomplishments and Results (by Objective)</u>:

Objective 1: not addressed yet

Objective 2: Mixed-stress cDNA libraries have been created for berries, leaves and roots of Chardonnay. Over 20,000 ESTs have been sequenced to date. Current libraries made with the

Uni-ZAP-XR cDNA synthesis kit and vector system (Stratagene, Inc.) have complexities of 1.5 x 10^6 to 3.5 x 10^7 pfu/µg DNA and average insert sizes ranging from 0.9-1.2 kb. Raw sequences were produced with a success rate of >99%. Following cleansing (removal of short, low quality, contaminated, and chimeric sequences) with our automated EST analysis pipeline (ESTAP) developed in collaboration with Jennifer Weller and Chunhong Mao at the Virginia Bioinformatics Institute (VBI; Blacksburg, VA), we attained an overall success rate was 88.25%. After cleansing, tentative functional assignments based on EST sequence similarity to related sequences in the non-redundant (NR) GenBank database were automatically obtained by BLAST searching. These results are summarized in figures 1 and 2. Approximately 44% and 55% of the sequences from leaf and berry EST collections, respectively, represent unknown or novel genes not identified in other organisms in the non-redundant (NR) GenBank database (Fig. 1). These results demonstrate the utility of our gene discover efforts in providing new molecular genetic information about wine grape. Furthermore, leaf ESTs displayed a higher proportion of significant similarity (56%) to known genes from other organism in the NR database than did berry ESTs (45%), probably due to the more intensive characterization of leaf gene functions relative to fruit gene functions within the plant biology community. More striking was the observation that berry ESTs exhibited a higher proportion of ESTs having no significant similarity match (no hits) to NR sequences (36%) compared to leaf ESTs (25%).

Leaf ESTs: **Berry ESTs:**

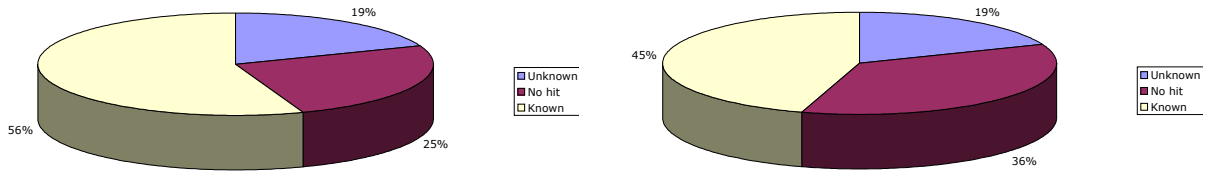

Figure 1. Sequence similarity of ESTs derived from leaf (left panel) and berry (right panel) to known genes in the NR database. This analysis was based on 5,730 leaf and 6,137 berry cleansed ESTs, respectively. See text for a discussion of these results.

Leaf ESTs: **Berry ESTs:**

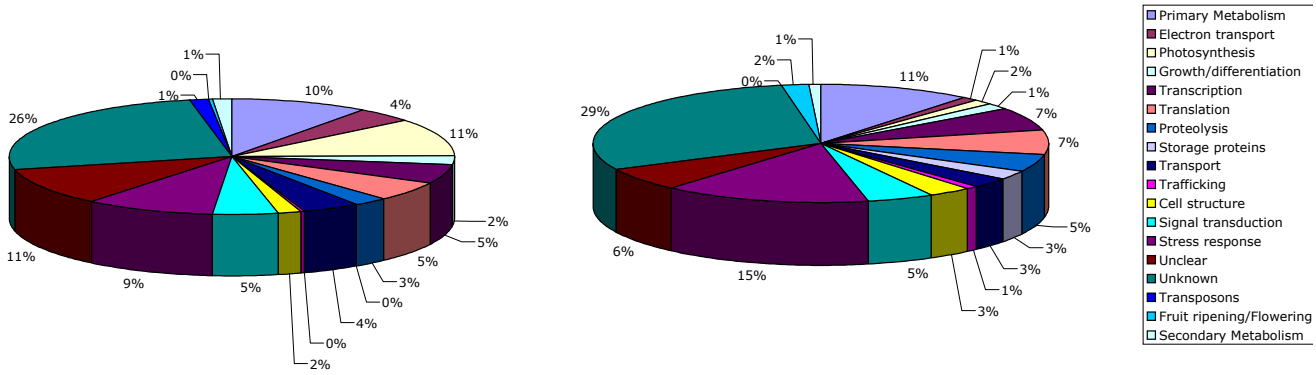

Figure 2. Functional assignment of ESTs derived from leaf (left panel) and berry (right panel) with significant matches to known genes in the NR database. This analysis was based on 4,297 leaf and 3,941 berry cleansed ESTs, respectively. See text for a discussion of these results.

Berry ESTs showed a higher percentage of novel sequences (no hit) (36%) compared with leaf ESTs (25%) presumably due to a greater complexity of genes recruited for secondary metabolism and specialized functions of berry development, ripening, and protection. Leaf and berry EST collections show distinct functional category assignment differences. As expected, berry ESTs showed a much lower percentage of photosynthesis-related genes (2%) compared with leaf ESTs (11%) (Fig. 2). Berry ESTs also showed a higher percentage of stress response genes (including biotic stress response functions) and fruit ripening/flowering-related genes than leaf ESTs (Fig. 2). In summary, a total of 11,867 ESTs from the leaf and berry cDNA libraries have been generated and deposited in GenBank and are freely available to the public at the NCBI dbEST (http://www.ncbi.nlm.nih.gov/dbEST/index.html) and ESTAP websites (http://www.vbi.vt.edu:8080/estap/servlet/Login). The leaf ESTs were assigned accession numbers BM436250-BM438127, CB035743-CB036198, and CB001075-CB004470. The berry ESTs have been assigned accession numbers CB004471-CB009714. An additional 9,500 EST have been generated from root cDNA libraries. These sequences are currently being subjected to a rigorous cleansing process to ensure removal of contaminating ESTs derived from soil microbial origin prior to deposition in dbEST. Not reported above are another 2,500 ESTs that have been sequenced or are in the process of being sequenced.

During the last year we have made several changes to our EST sequencing operations. First, we have switched to an in vitro template preparation technique using Templiphi (Amersham Pharmacia Biotech) that reduced our template preparation costs by 40% compared with alkaline lysis plasmid preparation using a Qiagen 3000 robot. Second, the Nevada Genomics Center recently upgraded the capillary array automated DNA sequencer from an Applied Biosystems Inc. (ABI) 3700 to an ABI 3730. The new instrument uses fewer reagents and consequently our sequencing costs have been reduced by approximately 15%. Both of these changes will allow us to reduce our EST production costs an average of 27% making our effort more efficient. In addition, the new 3730 sequencer has more than double the throughput capacity of the older instrument it replaced. We now have the capacity to sequence at least 894 templates per day. Our only limitation continues to be funding availability to conduct EST sequencing. We have also enhanced the capabilities of ESTAP. In addition to automated cleansing and database searching capabilities using BLAST, we have added both a keyword and local BLAST searching capability to search the local databases for discrete sets of sequences. We have also automated the clustering (cluster_2) and contig assembly (CAP3) procedures for the creation of "Unigene" (non-redundant) datasets. Such operations are critical for us to be able to rapidly and easily compile non-redundant datasets for the design of a publicly available oligonucleotide microarray in collaboration with the International Grape Genome Program (see VII. Research Success Statements). Finally, we are dissatisfied with the accuracy of annotations provided by BLAST searching. We are working to improve the quality of annotation information provided by ESTAP by automating the assignment of gene ontology information based on sequence motif searching provided by Interpro (http://www.ebi.ac.uk/interpro/). We expect the automated gene ontology assignment module of ESTAP to be operational within the next 6 months.

Objective 3: Not addressed yet.

Objective 4: Must samples were obtained from well-watered and drought-stressed Chardonnay grapes. Standards were developed for gas chromatography/mass spectroscopy in preparation for

must analysis. The standards included: 0.1, 0.5, 1.0, and 2.0 mg each of 2-methyl-1-propanol, 3-methyl-1-butanol, 2-phenylethanol, ethyl caproate, ethyl caprylate, diethyl succinate, hexanoic acid, octanoic acid, and decanoic acid to 200 mL of 10.5% ethanol. Standard solutions were injected into a gas chromatograph (Shimadzu gas chromatograph GC-14A equipped with a 60m x 0.25mm i.d. DB-WAX bonded phase, fused-silica capillary column and a flame ionization detector) in order to establish retention times and elution order for the different compounds and generate standard curves for must analysis. Preliminary comparative analyses of must samples show distinct differences in the composition and quantity of metabolites between well-watered and drought-stressed grapes. To improve our ability to conduct detailed metabolite analysis studies in the future, the Nevada Protemics/Metabolomics Facility recently took possession of a Finnigan Polaris-Q GLC/MS-MS (ion trap) mass spectrometer. This instrument will allow us to conduct detailed metabolite profiling and compound identification when analyzing for metabolite differences in must samples isolated from well-watered and drought-stressed berries.

VI. Outside Presentation of Research:

- Posters were presented at the 11th International Plant, Animal and Microbe Genomic conference held in San Diego in January 2003, at the Annual Meeting of the American Society of Plant Biologists, at the Annual Meeting of the American Society of Enology and Viticulture and at the Salt and Water Stress in Plants Gordon Conference, Oxford, England
- Dr. Cramer presented a talk entitled "Integrative Functional Genomics Resource Development in *Vitis vinifera*: Abiotic Stress and Wine Quality" at the International Grape Genome Project Workshop at the 11th International Plant, Animal and Microbe Genomic conference held in San Diego on January 12, 2003.
- Dr. Cushman present talks entitled "Towards Organizing Public Efforts on Grape EST Data and Microarray Design" at the International Grape Genome Project Workshop and "Integrative Functional Genomics of *Vitis vinifera*: Does Abiotic Stress Improve Wine Quality?" at the Abiotic Stress Workshop at the 11th International Plant, Animal and Microbe Genomic conference held in San Diego on January 12, 2003.

VII. Research Success Statements:

Mixed-stressed cDNA libraries for berries, leaves and roots have been made and EST sequencing has produced more than 20,000 ESTs to date. Raw sequences were automatically cleansed and annotated using an EST analysis pipeline (ESTAP) developed in cooperation with Chunhong Mao at VBI. Approximately 50% of the sequences are novel or lack informative annotation and sequence redundancy is currently at about 38%. All unique sequences identified to date (with the exception of the root EST collections) have been deposited in the GenBank dbEST database and are available to the public at the NCBI website: http://www.ncbi.nlm.nih.gov/dbEST/index.html. The annotated data are available to the public at the ESTAP website: http://www.vbi.vt.edu:8080/estap/servlet/Login

This grant has provided much needed seed money for the development of a much larger research initiative to conduct an integrative functional genomics project on abiotic stress and wine characteristics. The initiative includes an integration of genomic, proteomic and

metabolomic approaches. It also expands abiotic stress functional genomics research to Cabernet Sauvignon. The NSF Plant Genome program awarded us a $3.6 million grant for 4 years beginning in September 2002, largely because of the preliminary results obtain with funds from the American Vineyard Foundation and the USDA Viticulture Consortium.

The International Grape Genomics Consortium has developed a white paper that describes the current and future status for grape genomics on an international scale. We are active participants in the EST and transcriptional profiling and Bioinformatics working groups and hope to play a supportive role in the development of an international community database for EST information and DNA microarray gene expression data in collaboration with the Virginia Bioinformatics Institute and UC Davis. We have established an automated EST analysis pipeline (ESTAP) and associated database that serves as a main repository for our EST sequence information (and related gene expression information). The ESTAP database is currently linked with the International Grape Genome Program website (see http://www.vitaceae.org/) established by Dr. Doug Cook at the UC Davis College of Agricultural and Environmental Sciences. This site contains a database of researchers working on grape, resources for grape genomics, and links to other web sites relevant to *Vitis* various genomics resources.

We have invited other groups from the international wine grape research community with large EST collections to share their EST data with the aim that ESTAP will serve as a central repository for public EST data. Our goal is to provide a resource for the International Grape Genomics Community to compile EST data for the design of a first-generation oligonucleotide microarray for *Vitis vinifera*. The EST data obtained from this research (and other research efforts) will be automatically assembled into contigs and singlets to create a "Unigene" set for the design of oligonucleotides for this microarray. In collaboration with Dr. Cook's group at UC Davis (and other interested participants), we will fabricate the first *Vitis* oligonucleotide microarray containing approximately 12,000 unigenes by the summer 2003. Once completed, we plan to make this array publicly available at a minimal cost. The availability of this first generation oligonucleotide microarrays will permit detailed gene expression profiling studies to be conducted on a wide range of research topics related to grape biology and viticulture and may even be useful for enology related studies. This research will greatly facilitate future gene discovery and enable improvements to be made in both production efficiency and wine quality under environmentally adverse growing conditions.

VIII. Funds Status:

We have spent all available funds to complete all goals we were charged to complete. Additional fund are required to complete our next 10,000 EST sequences (our original goal was to sequence 30,000 ESTs over the course of the project) and fabricate a first generation oligonucleotide microarray.