INFORME TÉCNICO

Plant and Animal Genome XII Conference,
San Diego, CA.
10-14 Enero 2004

BID-FP-L-2003-2-BIOT-15

Inés Marlene Rosales Villavicencio

GOBIERNO DE CHILE
FUNDACIÓN PARA LA
INNOVACIÓN AGRARIA

INFORME TÉCNICO

PROGRAMA DE FORMACIÓN PARA LA INNOVACIÓN AGRARIA

1. Antecedentes Generales de la Propuesta

Nombre: Plant and Animal Genome XII Conference, San Diego, CA. 10-14 Enero 2004

Código: **BID-FP-L-2003-2-BIOT-15**

Nombre Postulante Individual: Inés Marlene Rosales Villavicencio

Lugar de Formación (País, Región, Ciudad, Localidad): EEUU, California, San Diego.

Fecha de realización: 10-14 Enero 2004

Objetivos de su participación en la actividad:
Actualización en los ámbitos de la genómica y bioinformática, así como en las nuevas áreas de investigación que actualmente se desarrollan en el ámbito internacional (metabolómica y proteómica).
Esto se traduce en concretar avances en los siguientes aspectos:

1. Vinculación internacional.
2. Posibilidad de acceder y utilizar resultados de investigación externa en Chile.
3. Capacitación en la tecnología de microarreglos, bioinformática y otras técnicas específicas.
4. Revisar experiencias internacionales en el ámbito de la propiedad intelectual.
5. Intercambio de experiencias en genómica y bioinformática.

2. Antecedentes Generales: describir si se lograron adquirir los conocimientos y/o experiencias en la actividad en la cual se participó (no más de 2 páginas).

Esta conferencia representa una de las instancias científicas más importantes en el área de la Genómica y Bioinformática. En ella se presentan año a año innumerables workshops y conferencias que muestran las últimas actualizaciones en éstas áreas. Para los proyectos financiados por la Iniciativa Genóma Chile, se presentó entonces una oportunidad única de interacción con otros grupos internacionales, que nos permitió conocer y aprovechar sus experiencias, así como también realizar una difusión del trabajo que en Chile se efectúa.

Sin duda, los objetivos planteados en término de actualización, vinculación internacional e intercambio de experiencias se cumplió a cabalidad. Sin embargo, este año se observó una clara ausencia de los grupos de investigación más sobresalientes de Europa y Australia, lo que afectó en particular a aquéllos interesados en los avances de la genómica en vides. Es posible que esta ausencia se deba a que en Junio próximo se realizará un encuentro Internacional en California-Davis, que congregará a los miembros del "Grape Genome Group", y éstos grupos estén privilegiando su asistencia a éste encuentro. A pesar de que este

Consorcio Internacional posee integrantes de distintos países, la cooperación Internacional que se observa es escasa y al parecer altamente competitiva.

3. **Itinerario Realizado**: entregar una relación de actividades de acuerdo al siguiente cuadro:

Fecha	Actividad	Objetivo	Lugar
8 al 9 de Enero, 2004	Vuelo Santiago-Miami-Dallas- San Diego	Arrivar a San Diego, CA	Santiago - San Diego
10-14 Enero 2004	Participación en Conferencia	Asistencia seminarios, workshops, exhibiciones, etc.	Town & Country Hotel, San Diego
15-16 Enero 2004	Vuelo San Diego-LA-Santiago	Retorno a Santiago, Chile	San Diego-Santiago

Señalar las razones por las cuales algunas de las actividades programadas no se realizaron o se modificaron.

Todas las actividades se cumplieron de acuerdo al itinerario programado por FIA.

4. Resultados Obtenidos: descripción detallada de los conocimientos adquiridos. Explicar el grado de cumplimiento de los objetivos propuestos, de acuerdo a los resultados obtenidos. Incorporar en este punto fotografías relevantes que contribuyan a describir las actividades realizadas.

Los conocimientos adquiridos durante este evento dicen relación con las conferencias presenciadas en este Congreso. La envergadura de esta Conferencia permite que en sus ponencias no sólo se presenten grupos de investigación líderes en el área de la Genómica y bioinformática, sino también las grandes compañías que entregan la tecnología necesaria para que estas áreas del conocimiento se desarrollen. A continuación se detallarán algunas sesiones de grupos de investigación líderes presenciadas, considerando como principal área de interés la Fruticultura. Los resúmenes referidos en el texto pueden encontrarse en http://www.intl-pag.org/12/abstracts/

1. **Fuit and Nut Crops Workshop.** Organizado por Erik Rikkerink (ERikkerink@hortresearch.co.nz). Horticulture and Food Research Institute of NZ Ltd.
En este workshop se presentaron investigaciones en genómica de frutales: cerezos, duraznos, papaya, manzanos y vides. En cerezos se presentó un trabajo en autoincompatibilidad gametofítica, la que se presenta en sour y sweet cherry (resumen W102).

Clemson University presentó su trabajo en genómica de duraznos, quienes en forma muy interesante están integrando la información de los mapas genéticos, físicos y la genómica funcional. Durazno aparece entonces como un modelo en frutales que presenta ventajas comparativas claras frente a otras especies: genoma de tamaño pequeño (300 Mb aprox), y un genoma diploide (2n= 16). Este grupo ya a generado 9984 ESTs de alta calidad, los

que han sido ensamblados y anotados definiendo de esta forma los primeros 3842 "unigenes" de duraznos, es decir un set de genes únicos, no representados en los genomas hasta ahora secuenciados de otros modelos vegetales. Este grupo posteriormente ha desarrollado un mapa de transcritos, para lo que hibridaron sus ESTs a librerias BAC de durazno, localizando 1304 ESTs de sus unigenes., es decir localizaron un 11.2% de sus ESTs en el mapa genético. Los datos que se generen en este proyecto y que sean de público acceso pueden encontrarse en http://www.genome.clemson.edu/gdr/. (resumen W104). La estrategia que este grupo ha desarrollado para su proyecto debiera servir de ejemplo en nuestras iniciativas nacionales, ya que la forma más eficiente de utilizar la información que se generen de proyectos de genómica funcional es acoplarlos con la información generada en mapeos físicos y genéticos de la especie en cuestión.

En manzanos se presentaron tres trabajos: conservación de marcadores alrededor de algunos genes en manzano; Silenciamiento de proteínas de manazana que interactúan con genes avr de *E.amylovora* (es decir potencialmente suprimirían la enfermedad), y aumento de la resistencia a enfermedades en manzanas que sobre-expresan el genMPNR1 (un mediador de la respuesta defensiva en vegetales) (resúmenes W100, W101 y WW105).

Finalmente, Pamela Gatto (IASMA-Italia) presentó la investigación en genómica de vides desarrollada en este Instituto. Este grupo, cuyo lider es Riccardo Velasco empezó el trabajo genómico en 1999, el que incluye un programa de secuenciación de ESTs, desarrollo de mapas moleculares , librerias BAC y mapeo físico así como aproximaciones genómicas tales como cDNA-AFLPs y macroarreglos. Al momento, han secuenciado 8000 ESTs, organizados en una base de datos que pronto se hará pública (www.ismaa.it) . Han encontrado 4400 unigenes, de los cuales 2300 han sido depositados en membranas de nylon, dónde se estudiará la regulación diferencial de genes involucrados en la ontogenia y senescencia de hojas de vides. Además, a través del uso de librerías substractivas, han podido generar arreglos tejido específico de hojas, frutos y raíces de vides. Con todo este material, se han concentrado en el estudio de la expresión global de genes de forma de poder ganar conocimiento en procesos tales como vías metabólicas de compuestos fenólicos y respuesta defensiva a *B.cinerea* y *P. vitícola*.

2. **Nacional Citrus Genomic Workshop**, organizado por José Chaparro del USDA/ARS.
Este workshop presentó una amplia variedad de trabajos. Primero, Gloria Moore mostró los intentos preliminares de la Universidad de Florida en el uso de las herramientas genómicas en el estudio de la tolerancia al frío que se presenta en *Poncirus trifoliata,* un "pariente silvestre" de los cítricos desde el cual es posible introgresar genes a la especie (Resumen W49). Más tarde, Gustavo Astúa-Monge presentó el gran despliegue que Brasil está realizando en esta área. Brasil ha iniciado hace algún tiempo un proyecto de secuenciación de ESTs enfocándose en la respuesta de cítricos a diferentes estrés bióticos y abióticos. Los datos evaluados se concentran en el descubrimiento de microsatélites, marcadores SNPs e identificación de genes diferencialmente expresados bajo estas condiciones. De esta forma, el trabajo bioinformático les ha permitido encontrar agrupaciones de genes (gene clusters) que presentan respuestas similares a diferentes formas de estrés que se han evaluado. Un destacable ejemplo de trabajo multiprofesional de avanzada en nuestro hemisferio, sin duda uno de los más importantes que se presentaron en el evento (resumen W50).

Posteriormente dos grupos americanos presentaron sus trabajos en Citrus ESTs Project, donde se mostraron las abundantes colecciones de ESTs que han obtenido bajo distintas condiciones de forma de representar la mayor cantidad de unigenes posibles. El grupo de California (M.Roose, resumen W52) ha fabricado 12 librerías principalmente desde naranja dulce, las que en el futuro se dispondrán en un microarreglo de cítricos. Este trabajo aún está en marcha y sólo se presentó trabajo bioinformático de genes candidatos. R. Shatters del USDA en Fort-Pierce, FL (resumen W53) ha centrado el análisis de ESTs en condiciones de interacción planta-insecto, de forma de dilucidar algunas interacciones de insectos vectores, mosquitas blancas, etc con la planta. Finalmente, Vicente Conejeros y Manuel Talon de España presentaron su colección de más de 30 mil árboles de cítricos que han sido obtenidos después de realizar mutagénesis al azar, y la colleción de ESTs que serán utilizados en estudios de genómica funcional en Valencia (resúmenes W51 y W 55).

Finalmente, Eric Mirkov, de Texas A&M University presentó el trabajo de clonamiento del gen de resistencia al virus de la tristeza de los cítricos. Este grupo ha definido, por medio de mapeo físico y genético, un área de 300kb que contiene el gen de interés, pero en el que han encontrado 12 posibles genes de resistencia a enfermedades. En estos momentos se encuentran realizando trabajos de complementación en variedades susceptibles (vía transformación genética) de forma de encontrar cuál de estos genes es efectivamente el gen de resistencia a este virus.

2. Internacional Grape Genome Project Workshop, organizado por Douglas Cook Universidad de California (drcook@ucdavis.edu).

John Cushman (University of Nevada, Reno) presentó detalles del microarreglo que Affymetrix está preparando para ser utilizado por algunos miembros del Consorcio de Vides. Para la confección del mismo, se están utilizando las secuencias de ESTs que han sido liberadas al dominio público por distintos grupos de investigación. Los chips de Affymetrix han sido documentados como los más confiables y reproducibles en el mercado, por lo esto significa un avance importante para aquellos interesados en trabajar en genómica de vid. En el caso de los investigadores nacionales, el grupo que trabaja en la Plataforma Científica y Tecnológica para el Desarrollo de la Genómica en Chile: Etapa I Vid, (iniciativa en la que INIA está incluída) no participó en el Consorcio que financió la puesta en marcha de esta iniciativa, debido al alto costo involucrado. Para acceder a ellos en el futuro cercano, se deberá negociar directamente con los grupos en Reno y California, quienes definirán el precio de los usuarios "menores" de este microarreglo. Durante este encuentro se especuló de un costo de $500/arreglo, lo que no consideraba los softwares para decodificar la información que entregue el microchip, servicios que son prestados por la misma empresa Affymetrix.

Casi en paralelo durante esta conferencia, se conoció que existía una iniciativa Europea, que había encargado a la empresa Qiagen, la confección de un set de oligonucleótidos que pueden ser utilizados en un formato muy similar al que emplea Affymetrix en sus diseños. Aún no se conocen detalles técnicos en cuánto a la confección, diseño y comportamiento de estos oligos ya que aún no han sido utilizados por los investigadores.

Posteriormente, tres grupos norteamericanos de Reno y California presentaron un análisis de proteómica en vides estresadas por déficit de agua, estrés salino y estrés por nitrógeno (Resúmenes P765, P766 y P41). Por otra parte, D. cook presentó información respecto a respuestas transcripcionales de vid frente a infección por la bacteria del xylema *Xylella*

fastidiosa. Con respecto a ésta última los avances presentados fueron escasos con respecto al material ya informado en previos meetings o publicaciones (resumen P40).

En general, lo más interesante de esta jornada fue conocer detalles del microarreglo de vides que pronto estará disponible a la comunidad científica. Los miembros del consorcio internacional del genoma de la vid no presentaron avances significativos en sus ponencias, lo que no necesariamente significa que éstos no están ocurriendo al interior de los conglomerados. Una fuerte competencia con el bloque Europeo parece ser la razón para la confidencialidad, además de una próxima Conferencia en California-Davis, que juntará a los expertos del tema.

Ya que es imposible detallar todas las ponencias presenciadas durante los 5 días del evento, se han privilegiado en este resumen aquellas cercanas al área de acción de la becada. En cuanto a las nuevas tecnologías presentadas, las más interesantes fueron sin duda los workshops organizados por Affymetrix, Whatman y Sigma.
En cuanto al cumplimiento de los objetivos planteados inicialmente, éstos se cumplieron en términos de actualización, capacitación y revisión de las experiencias internacionales. El intercambio de experiencias no es siempre tan fácil de acceder, ya que existen intereses, restricciones económicas y de propiedad que juegan un papel relevante ante la posibilidad de compartir información. Dentro de lo posible, se trató de conocer de las experiencias de otros grupos para así aprovecharlas dentro de la realidad local.

5. Aplicabilidad: explicar la situación actual de los temas en Chile (región), compararla con la tendencias y perspectivas en el país (región) y feria visitados y explicar la posible incorporación de los conocimientos adquiridos, en el corto, mediano o largo plazo, los procesos de adaptación necesarios, las zonas potenciales y los apoyos tanto técnicos como financieros necesarios para hacer posible su incorporación en nuestro país (región).

Sin duda que otros países ya han desarrollado el tema de la genómica y bioinformática por adelantado. En nuestro país se está recién comenzando en este tipo de trabajo, pero en lo posible, se ha tratado de aprovechar las experiencias internacionales, como por ejemplo, evitar los errores cometidos al momento de confeccionar las librerías, elección de plataformas a utilizar, estrategias bioinformáticas más apropiadas, etc.
Muchos de los grupos internacionales de vanguardia ya han pasado a otro tipo de análisis, como es la proteómica y metabolómica, caminos que sin duda recorreremos en el futuro como nación. Por ahora, pareciera ser que la lección más importante que se saca es la necesidad de contar con mapas físicos y genéticos adecuados, que permitan utilizar a cabalidad los resultados de la genómica funcional. En INIA, estamos trabajando en ese sentido, ya que estamos involucrados en la investigación en areas de mapeo genético, transformación y genómica en vides. Nos queda por lo tanto equiparar estos esfuerzos en frutales de carozo, dónde nuestra Institución ya se ha integrado al trabajo en genómica y transformación genética de *Prunus*.
Ya que los apoyos financieros por parte de la Iniciativa Genoma Chile fueron asignados por tres años, se hace necesario pensar con anticipación en la continuación de la investigación en el área de la genómica y bioinformática en Chile, de forma de aprovechar lo resultados de estos tres años de trabajo.

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6. Contactos Establecidos: entregar una relación de contactos establecidos de acuerdo al siguiente cuadro:

Institución/Empresa	Persona de Contacto	Cargo/Actividad	Fono/Fax	Dirección	E-mail
(Horticulture and Food Research Institute of NZ Ltd.	Erik Rikkerink	Investigador			ERikkerink@hortresearch.co.nz
Universidad de California . Comité del Internacional Grape Genome Project	Douglas Cook	Investigador			drcook@ucdavis.edu
University of Nevada, Reno. Comité del Internacional Grape Genome Project	John Cushman	Investigador	(775) 784-1918 (775) 784-6258	Fleischmann Agriculture Building, Office 307	jcushman@unr.edu
Centro APTA Citros Sylvio Moreira, Cordeiropolis, SP-Brasil	Gustavo-Astúa Monges	Investigador Biotechnology Lab.			gamo@centrodecitricultura.br

Se destacan algunos de los contactos establecidos. Los tres primeros vinculados al consorcio internacional de genómica en vides y el cuarto, un investigador residente en Brasil, trabajando en genómica en cítricos, que representa a uno de los grupos sudamericanos más importantes que se presentaron en este evento.

7. Detección de nuevas oportunidades y aspectos que quedan por abordar: señalar aquellas iniciativas detectadas en la actividad de formación, que significan un aporte para el rubro en el marco de los objetivos de la propuesta, como por ejemplo la posibilidad de realizar nuevos cursos, participar en otras ferias y establecer posibles contactos o convenios. Indicar además, en función de los resultados obtenidos, los aspectos y vacíos tecnológicos que, a la luz de los conocimientos adquiridos en esta actividad, aún quedan por abordar para la modernización del tema en el país.
Se destacarán tres puntos importantes que quedaron en claro después de asistir a esta conferencia:
a. Existirá una próxima reunión del grupo especialista en vides a efectuarse en CA-Davis en Junio próximo dónde se espera encontrar avances significativos en los áreas de la genómica, proteómica y metabolómica
b. La confeccion de microarreglos de vides por la empresa Affymetrix podría redireccionar la estrategia planteada hasta ahora por el Consorcio de vides chileno, en cuanto a utilizar sus microarreglos v/s el uso de macroarreglos nacionales. Todo dependerá de los costos implicados y las capacidades nacionales.
c. Se hace necesario y urgente avanzar en los temas de mapeamiento físico y genético en los frutales modelos de la genómica nacional (vides, carozos).

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8. Resultados adicionales: capacidades adquiridas por el participante individual y/o el grupo, como por ejemplo, formación de una organización, incorporación (compra) de alguna maquinaria, desarrollo de un proyecto, firma de un convenio, etc.
Se contactaron múltiples proveedores que usualmente son utilizados por nuestros laboratorios de forma de estudiar vías más expeditas y económicas de acceder a sus servicios (síntesis de óligonucleótidos, secuenciación de ADN, membranas y filtros, etc).

9. Material Recopilado: junto con el informe técnico se debe entregar un set de todo el material recopilado durante la actividad de formación (escrito y audiovisual) ordenado de acuerdo al cuadro que se presenta a continuación (deben señalarse aquí las fotografías incorporadas en el punto 4):

Tipo de Material	Nº Correlativo (si es necesario)	Caracterización (título)
Reproducción de poster	1	Cloning and characterization of flower development genes in papaya
Reproducción de poster	2	Candidate gene database and transcript map for peach: a model genome species for *Rosaceae*
Reproducción de poster	3	Comparative analysis of ethylene response factors and pathogenesis related proteins in *Vitis vinifera* and *Vitis aestivalis*
Reproducción de poster	4	Identification of disease defense- and stress- related genes in *Vitis shuttleworthii* grape through EST analysis
Reproducción de poster	5	Molecular characterization of genetic variability and differentiation among European and Asian grape cultivars
Reproducción de poster	6	A fully automated system to extract DNA from difficult plant tissues
Reproducción de poster	7	Microarray analysis of defense against Sclerotinia stem rot in soybean

Cloning and characterization of flower development genes in papaya

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Introduction

Papaya (*Carica papaya* L.) is a polygamous species with three basic sex forms: female, male, and hermaphrodite. Although those three sex forms are genetically determined (Hofmeyr, 1938), the phenotypic sex expression of papaya is influenced by environmental factors, including temperature (Awada, 1958), nutritional status (Awada and Ikeda, 1957) and moisture (Awada, 1961). Instability of papaya flower sex expression is common, and sex reversal occurs in all three sex forms of papaya flowers, especially in hermaphrodite and male flowers (Storey, 1958). Incomplete sex reversal results in a continuous graded series of flower types (Storey, 1958). Thus, papaya provides a unique opportunity to study flower development in plants. On the other hand, the instability of papaya flowers can result in unmarketable, malformed fruit. Cloning major genes controlling flower development in papaya is the first step towards solving this problem. According to the widely accepted "ABC" model, "C" function genes are required for both stamen and carpel formation. *Agamous* (*AG*) is the only "C" class gene in *Arabidopsis*. We report here the cloning of "C" function gene in papaya, *PAG*, and two of its regulatory genes, *PFL* and *PHUA*1.

Materials and Methods

Plant material. Four different papaya cultivars, SunUp, Kapoho, Saipan Red, and Drew, were germinated and planted in Kunia station, Oahu. Young leaf tissues of SunUp, Kapoho, and Drew were collected for genomic DNA isolation. Hermaphrodite and female flower buds, young leaf tissues, and root tissues were collected for total RNA isolation. Shoot apical meristem, floral meristem, and leaf meristem tissues from SunUp, Kapoho, and Saipan Red were collected and fixed at different floral developmental stages for *in situ* hybridization.

cDNA library construction. Total RNA was isolated from hermaphrodite and female flower buds. PolyA+ RNA was isolated from the total RNA using Streptavidin Magnesphere particles (Promega). Double stranded cDNA was synthesized using ZAP-cDNA Synthesis kit (Stratagene) and cloned into ZAPII vector (Stratagene).

Screening of BAC, cDNA library and sequencing. *Arabidopsis LEAFY*, *AG* (provided by Elliot Meyerowitz), and *HUA*1 (provided by Xuemei Chen) cDNA clones were used to screen the papaya BAC and hermaphrodite and female flower cDNA libraries. The positive cDNA clones were sequenced directly using universal primers. Complete genomic sequences were obtained from sequencing positive BAC clones beginning with the primers designed in the subclone sequences by primer walking.

Southern blot analysis. Papaya genomic DNA of three different sex forms was digested with *EcoR* I, *Hind* III, and *Xba* I and transferred onto Nylon membrane. Standard methods were used for Southern blotting and hybridization.

Northern analysis. Total RNA was isolated from roots, leaves, flower primodium, mature flowers, and carpels. Standard methods were used for Northern blotting and hybridization.

In situ hybridization. *In situ* hybridization was performed by using digoxigenin (DIG)-labeled RNA probes according to the manufacturer's instruction (Boehringer Mannheim).

Results and Discussion

Using *Arabidopsis LEAFY*, *AG*, and *HUA*1 cDNA clones as probes to screen papaya BAC and cDNA libraries, *LEAFY*, *AG*, and *HUA*1 homologous genes in papaya, *PFL*, *PAG*, and *PHUA*1, were cloned and sequenced. *PAG* has nine exons and eight introns (Figure 1a) and encodes a putative protein with 228 amino acids. *PAG* protein shares 69% identity and 79% similarity with *Arabidopsis AG*. Within the MADS region, *PAG* shares greater than 98% amino acid sequence identity (only one amino acid difference) with *Arabidopsis AG*. The 2nd intron of *Arabidopsis AG* is about 3kb, and contains functionally important regulatory sequences for *AG* expression (Hong et al., 2003). In papaya, the 2nd intron of *PAG* is about 6-7kb, much larger than that of *Arabidopsis*. Genomic and BAC Southern analysis indicate that *PAG* exists as a single copy in the papaya genome (data not shown). Northern analysis showed that *PAG* is expressed preferentially in floral tissue, since no signal was detected in leaf and root tissues (Figure 2), *PAG* begins its expression from a very early stage of flower development and continues through mature flower where it is highly expressed in the carpel (Figure 2).

PFL has three exons and two introns (Figure 1b) and encodes a protein of 367 amino acids. The comparison of *PFL* protein to other *LEAFY*-like proteins showed that *PFL* protein is more similar with other dicot *LEAFY*-like proteins than with their monocot counterparts or with gymnosperm *LEAFY*-like proteins. *PFL* protein shares 61% identity and 66% similarity with the *Arabidopsis LEAFY* protein (Table 1). Unlike its *LEAFY* counterparts from other plant species, *PFL* lacks the proline-rich region that was found in most *LEAFY*-like proteins. This difference may not affect the gene function as demonstrated by research on the *Pinus radiata LEAFY* homolog *Needly* (Mouradov et al., 1998). Genomic and BAC Southern analysis showed that papaya has only one copy of *PFL* (data not shown). The *in situ* hybridization result revealed that *PFL* mRNA was already detected in the shoot apical meristem (SAM) of young seedlings of all three papaya genotypes, Kapoho, SunUp, and Saipan Red, at 5-node stage (Figure 3a). *PFL* mRNA was also detected at a very high level in flower primodia (Figure 3d) and at a relatively lower level in leaf primodia (Figure 3b). Expression of *PFL* was observed in the adaxial face and margins of leaf (Figure 3b) and was also detected in developing floral organs but at lower levels than in flower primodia (Figure 3c).

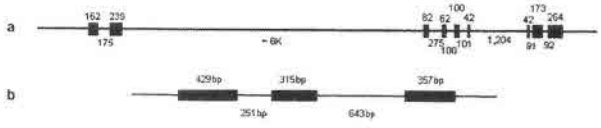

Figure 1. a. Genomic organization of *PAG*. b. Genomic organization of *PFL*. The exons are shown as black boxes and introns are shown as horizontal lines. The sizes of exons and introns are given above or below them.

Table 1. Comparison of amino acid sequences between papaya and *Arabidopsis* homologous genes

Homologous Genes	Percentage of identity	Percentage of similarity
PFL	61%	66%
PAG	69%	79%
*PHUA*1	62%	73%

*PHUA*1 cDNA is about 1.8kb and encodes a putative protein with 522 amino acids. The deduced amino acids sequence shares 62% identity with *Arabidopsis HUA*1 (Table 1) and the six tandem CCCH-type zinc finger motifs are highly conserved between *HUA*1 and *PHUA*1.

The plant MADS box genes share a similar structure, consisting of a highly conserved MADS box domain and a moderately conserved K domain. This high level of conservation causes difficulties for the identification of different subfamilies of MADS genes. Based on the cDNA sequence, *PAG* shares a higher identity with *SHP*1 and *SHP*2 than with *AG*. However, *PAG* contains 9 exons and a huge 2nd intron, which matches *Arabidopsis AG* exactly. In contrast, *SHP*1 and *SHP*2 have 7 and 6 exons, respectively. In addition, both of *SHP*1 and *SHP*2 have smaller 2nd intron, 1.3kb and 2kb in length, respectively. Moreover, Southern analysis indicated that the papaya genome does not contain additional *AG* related genes. The 2nd intron of *Arabidopsis AG*, which contains *cis* regulatory domains of *AG*, is critical for its normal spatial and temporal expression. In papaya, the 2nd intron of *PAG* is about 6-7kb, much larger than that of *Arabidopsis*. It is not clear if the 2nd intron of *PAG* contains more complex regulatory elements than that in *AG*.

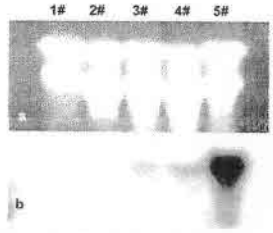

Figure 2. a. The gel image of total RNA from different papaya tissue. 1#: total RNA from papaya root. 2#:total RNA from papaya leaf. 3#: total RNA from papaya flower primodia. 4#:total RNA from papaya mature flower. 5#: total RNA from papaya mature carpel. b. Northern film showing *PAG* expression pattern.

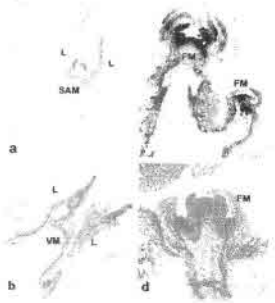

Figure 3. *In situ* hybridization of *PFL* transcripts in vegetative and floral tissues of papaya. a. Longitudinal section of shoot apical meristem(SAM). Expression was detected in the SAM and in the internal face and margins of leaves (L). b. Longitudinal section of vegetative meristem. Expression was observed in vegetative meristem (VM) and leaf primodia. c. Longitudinal section of a floral branch meristem (FM). Hybridization signals were observed in floral meristems (FM) at different developmental stages. d. Longitudinal section of inflorescence. Expression is detected in all floral organ primodia.

Genetic and molecular study of the expression pattern of *AG* in monoecious plants has revealed a complex set of positive and negative regulators that assure the stamens and carpels develop at appropriate time and in the appropriate floral regions. *LEAFY* is one of the positive regulators of *AG*. *PFL*, the *LEAFY* homologous gene in papaya, is highly expressed throughout the whole flower primodium that would develop into four different flower organs. At a relatively late stage of flower development when sepals and petals have been formed and stamens and carpels are still under development, *PFL* expression is much higher in the inner whorls of the flower than in the outer whorls. This suggests that *PFL* could be a regulator of papaya floral organ identity genes. Based on the partial sequence of the 2nd intron of *PAG*, five potential *LEAFY*-like protein-binding sites (CCANTG) were found within the 2nd intron of *PAG*. Thus, *PFL* may act as a positive regulator of *PAG* in papaya such as the case in *Arabidopsis*.

Reference

Awada, M. (1958). Relationships of minimum temperature and growth rate with sex expression of papaya plants (Carica papaya L.). Hawaii Agricultural Experiment Station Technical Bulletin 38, University of Hawaii, Honolulu.

Awada, M. and Ikeda, W.S. (1957). Effects of water and nitrogen application on composition, growth, sugars in fruits, yield, and sex expression of the papaya plants (Carica papaya L.). Hawaii Agricultural Experiment Station Technical Bulletin 33, University of Hawaii, Honolulu.

Cheng, Y., Kato, N., Wang, W., Li, J., and Chen, X. (2003). Two RNA binding proteins, *HEN*4 and *HUA*1, act in the processing of *AGAMOUS* pre-mRNA in *Arabidopsis thaliana*. Developmental Cell 4, 53-66.

Hong, R.L., Hamaguchi, L., Busch, M.A., and Weigel, D. (2003). Regulatory elements of the floral homeotic gene *AGAMOUS* identified by phylogenetic footprinting and shadowing. Plant Cell 15, 1296-1309.

Mouradov, A., Glassick T., Hamdorf, B., Murphy, L., Fowler, B., Marla, S., and Teasdale, R.D. (1998). *NEEDLY*, a *Pinus radiata* ortholog of *FLORICAULA/LEAFY* genes, expressed in both reproductive and vegetative meristems. PNAS 95, 6537-6542.

Storey, W.B. (1958). Modifications of sex expression in papaya. Hort. Adv. 2, 49-60.

CANDIDATE GENE DATABASE AND TRANSCRIPT MAP FOR PEACH: A MODEL GENOME SPECIES FOR ROSACEAE

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Goal

Peach (*Prunus persica* [(L.) Batsch] represents a model species for the Rosaceae, which includes a number of economical important fruit trees. Therefore, we have initiated a peach EST project with the goal of developing an extensive peach EST database and transcript map for identification and cloning of genes important to fruit and tree development.

Generation and Assembly of Fruit ESTs

We 5'-sequenced 13,331 clones from a peach cDNA library of developing fruit mesocarp (doupled haploid selection P21-5-2N) with an overal success rate of 75%, calculated after removal of poor quality and vector sequences. This resulted in 9984 succesful reads with an average length of 502 bp. These sequences were submitted to NCBI GenBank dbEST (accession numbers BU039022 through BU49005). The ESTs were assembled into contigs and singletons to define the first putative unigene set of peach consisting of 3842 ESTs.

Functional Annotation of Fruit ESTs

We characterized the *Prunus persica* EST sequences with respect to functionally annotated genes in the SWISS-PROT database. Of the 1552 sequences from the putative peach unigene set that had matches with the SWISS-PROT database, 1439 could tentatively be assigned Gene Ontology (GO) classifications (Fig. 1) based on the single „best hit" match against SWISS-PROT database (<1e-9). Functional assignments of peach ESTs described here are at the „inferred from electronical annotation" (IEA) level of evidence (see The Gene Ontology Consortium). Figure 1 summarizes assignments of peach sequences to major molecular functions and biological processes.

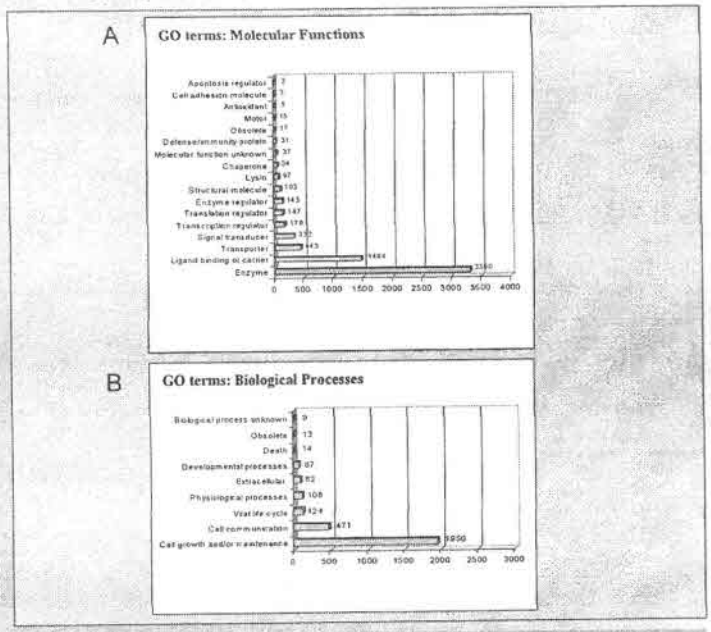

Fig. 1: General statistics for the number of proteins in the peach proteome that were assigned to the gene ontology terms A. "Molecular functions" and B. "Biological Processes". ESTs may be assigned to more than one GO term. Also note that child terms (not shown) may have more than one parent term (e.g. "hydrolase/

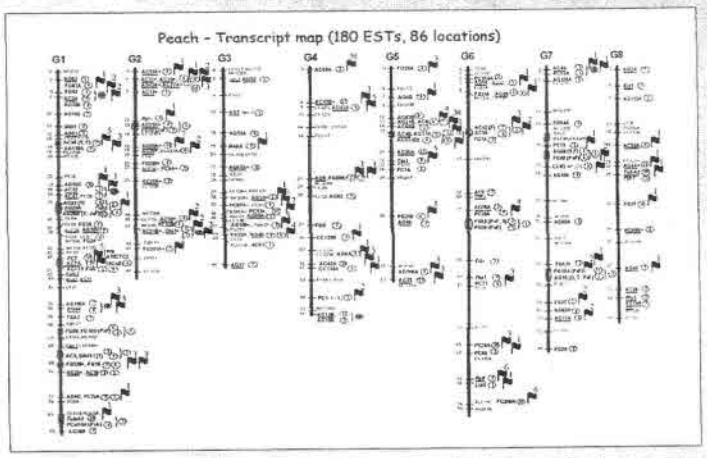

Fig. 2: Transcript map based on the general *Prunus* genetic map (Joobeur et al. 1998). Markers depicted in bold have been used to develop contigs of peach BACs (number of BACs in the light colored circles). Green circles denote adjacent markers detecting the same BACs. Black flags behind the markers represent peach EST positions. The number at the flags gives the number of ESTs mapped to this marker.

Development of a Transcript Map

Using 153 core markers from the general *Prunus* genetic map, BAC clones were anchored on the map providing a framework for the construction of a physical and a transcript map. To develop a transcript map, 1552 ESTs (PP_LE) from the putative unigene set and additional 68 peach cDNA clones (cultivar Loring) were hybridized against the peach BAC library. Hybridizing ESTs to genetically anchored BACs immediately localized 180 ESTs (11%) to 86 locations on the genetic map. ESTs showed clustering of expressed genes in defined regions of the linkage groups.

Access to the *Rosaceae* Genome Database

All data are incorporated into the *Rosaceae* genomics website ( ) regularly updated at Clemson. The database can be searched for BACs, ESTs, Markers and Maps (Fig. 3). Additionally, all our *Prunus* structural and functional genomics resources such as BAC libraries (peach, cherry, plum, apricot) and EST unigene libraries (peach, almond) are publicly available through the Clemson University Genome Institute.

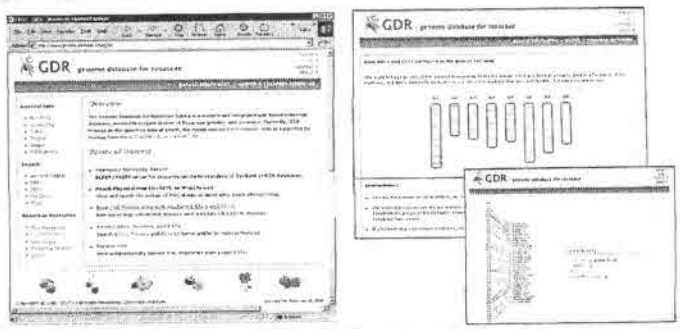

Fig. 3: Website of the database on Clemson University server.

Comparative Analysis of Ethylene Response Factor and Pathogenesis-Related Genes in *Vitis vinifera* and *Vitis aestivalis*

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Vitis Gene Discovery Program

Abstract:
Ethylene response factors (ERFs) are key regulators of the plant's response to pathogen attack. ERFs regulate an array of defense-related genes, such as those encoding pathogenesis-related (PR) proteins, by interacting with specific DNA elements (GCC boxes) in their promoters. We are interested in the characteristics and expression of these genes in the disease-resistant and susceptible grape varieties, *Vitis aestivalis* 'Norton' and *Vitis vinifera* cv. Cabernet Sauvignon, respectively. We identified and cloned cDNAs of several *V. aestivalis* ERF and PR genes that were previously demonstrated to play a role in defense in other plant species. In this study, we performed comparative analyses of three ERF and two PR genes at the sequence and transcriptional expression level. Results of these studies indicate that the expression of ERF genes were similar in leaves of both *V. aestivalis* and *V. vinifera* following treatment with ethylene, methyl jasmonate, or salicylic acid. In contrast, PR genes endochitinase IV and a thaumatin-like protein were expressed significantly higher in *V. aestivalis*. The expression of these ERF and PR genes was clearly elevated in powdery mildew infected leaves relative to non-symptomatic leaves, but the level of expression of the genes were considerably higher in *V. aestivalis* than in *V. vinifera*. In light of these results, it is tempting to speculate that an elevated expression level of defense-related genes may be a reason why *V. aestivalis* is more resistant to grape diseases.

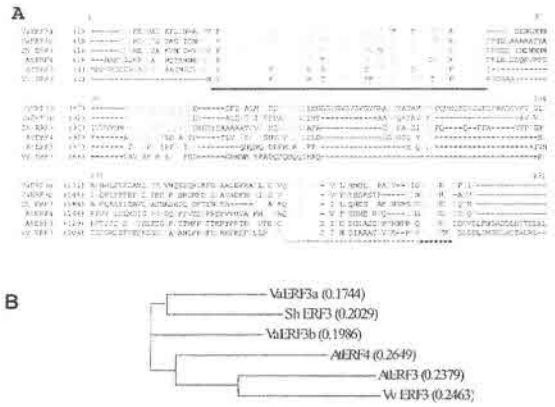

Figure 1. Amino acid sequence alignment (**A**) and phylogenetic relatedness (**B**) of two putative *V. aestivalis* 'Norton' ERFs (VaERF3a and VaERF3b) with the best-matched orthologs of *Stylosanthes hamata* (ShVRF3), *Arabidopsis thaliana* (AtERF3 and AtERF4) and *V. vinifera* (VvERF3). Amino acids that constitute the ERF domain are underlined. ^, acidic domains; +, putative MAP kinase target sites; *, ERF-associated amphiphilic repression (EAR) motif.

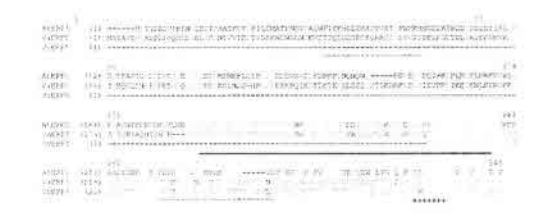

Figure 2. Alignment of predicted amino acid sequence of *V. aestivalis* VaERF5 with orthologues from *A. thaliana* (AtERF5) and *V. vinifera* (VvERF5). For legend, see Fig 1.

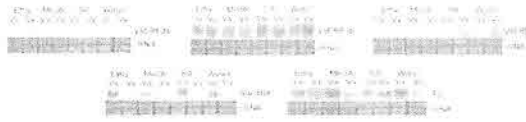

Figure 3. RNA gel blot analysis of VaERF3a, VaERF3b, VaERF5, VaCHI4 and VaTL in the leaves of *V. aestivalis* and *V. vinifera* following treatment with ethyphon (Ethy), methyl jasmonate (MeJA), and salicylic acid (SA). Greenhouse-grown grapevines were treated by spraying their fully expanded leaves on both sides with 1mM Ethy, 0.1mM MeJA, 2mM SA, or water (as control). Leaves were harvested 6 h after treatment for RNA purification. Each lane contains 20 µg of total RNA. Va, *V. aestivalis*; Vv, *V. vinifera*. Ethdium bromide stained images of rRNA bands underneath the autoradiographs indicate the relative amount of total RNAs loaded. Two biological replicates produced similar results.

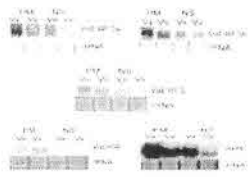

Figure 4. RNA gel blot analysis of VaERF3a, VaERF3b, VaERF5, VaCHI4 and VaTL in powdery mildew-infected and non-symptomatic leaves of *V. aestivalis* and *V. vinifera*. Powdery mildew-infected leaves were collected from naturally-infected greenhouse-grown vines.

Each lane contains 20 µg of total RNAs. PM, powder mildew infected; NS, non-symptomatic. Ethidium bromide stained images of rRNA bands underneath the autoradiographs indicate the relative amount of total RNAs loaded. Two additional biological replicates produced similar results.

Results and Conclusions:

1. Both VaERF3a and VaERF3b possess the functional domains that are represented in AtERF3, although they differ from one another in their predicted amino acid sequences (Fig. 1 A and B). Each VaERF3 contains an amphiphilic repression (EAR) motif, suggesting that they may function as transcriptional repressors.
2. The expression level of VaERF3b was high regardless of the treatment or the *Vitis* species. In contrast, the expression of VaERF3a couldn't be induced by Ethy, MeJA, or SA treatment in either species. Thus, VaERF3b is likely to be expressed constitutively, while VaERF3a appears to be inducible.
3. The expression of VaERF5 (Fig. 2) showed no up-regulation in response to treatments and did not differ between grape species (Figs. 3). This gene may maintain a constitutive base-level expression of defense-related genes, such as PR protein genes.
4. VaCHI4, a PR gene, was highly expressed in all treatments, including control in *V. aestivalis*, but could not be detected in *V. vinifera* (Figs. 3). The expression pattern of VaTL was similar to that of VaCHI4, but differed in its obvious response to SA in *V. vinifera* (Fig. 3).
5. All ERF and PR genes included in this study were expressed at a considerably higher level in powdery mildew-infected leaves relative to non-symptomatic leaves in both grape species (Fig. 4).
6. Both VaCHI4 and VaTL were highly expressed in *V. aestivalis* in both treated and infected leaves despite the similar expression of the ERFs in *V. aestivalis* and *V. vinifera*. These results suggest that the basal expression levels of some PR genes are higher in *V. aestivalis* than in *V. vinifera*. This may partially explain high disease resistance in *V. aestivalis* 'Norton'.

Identification of Disease Defense- and Stress –Related Genes in *Vitis Shuttleworthii* Grape through EST Analysis

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ABSTRACT

An Expressed Sequence Tags (EST) project aiming to explore disease defense and stress – related genes in *Vitis shuttlewothii* grape has been initiated at the Center for Viticulture and Small Fruit Research, Florida A&M University. *V. shuttleworthii* is a grape species native to the southeast United state and possesses resistance to major grape diseases and pests. A total of 12,936 ESTs produced from leaves and flowers during anthesis were sequenced, and of which 12,008 clean ESTs were generated after vector/end-trimmed using Squencher 4.17 program. Sequence analysis revealed that 18% of the *V. shuttleworthii* ESTs are unique when compared to the existing *Vitis vinifera* NCBI data bases, and 13% of the ESTs did not find any homologous sequences among all plant ESTs reported in NCBI. Overall, approximately 7% of the ESTs were related to disease/pest defense or stress tolerance genes. A total of 2106 contigs were assembled, and over 50 of which were identified as full-length cDNAs. The genome-wide identification/isolation of disease defense- and stress-related genes from *Vitis shuttleworthii* grapes will provide important genes and genetic markers for grape variety improvement.

INTRODUCTION

Vitis shuttleworthii is one of the most disease and pest resistant grape species which originated and is distributed in the southeast United States. It is highly resistant to: Pierce's Disease, Anthracnose, Black rot and Downy mildew diseases, which are the limited factors to grow *Vitis vinifera* grapes in this region. Clones of *V. shuttleworthii* have been extensively used by grape breeders in this region for developing disease resistant cultivars to combat diseases, pests, and environmental stresses. For these reasons, we selected *V. shuttleworthii* accession JL 2001 for our EST program for potential gene discover and marker development.

MATERIALS AND METHODS

cDNA libraries were constructed from mRNA isolated from leaves and flowers harvested during anthesis. Library construction and DNA sequencing were conducted in USDA-ARS Horticultural Research Lab, Fort Pierce, FL. Vector and end trimming were performed with Sequencher 4.17b (Gene Codes, Ann Arbor, MI) with manual editions for abnormal sequences. ESTs were subjected to similarity searches against the GeneBank non-redundant (nr) protein and EST databases. Sequence matches with *E*-value score <= 10^{-20} were considered significant. ESTs were assembled with a 95% minimum match and 50 base minimum overlap as assembly parameters.

Table 1. *V. shuttleworthii* EST project summary

Library and EST summary	
Number of cDNAs sequenced	12,936
Mean raw EST length	583 bases
Number of high quality ESTs	12,008
Mean clean EST length	521 bases
Contig assembly results	
Number of ESTs assembled	12,008
Number of contigs	2106
Number of sigletons	3670
Number of assembled sequences	5776
Summary of contigs	
Maximum size	2648 bases
Minimum size	216 bases
Maximum number of ESTs	85
>12 ESTs	94
9-11 ESTs	51
6-8 ESTs	139
3-5 ESTs	407
2 ESTs	1415

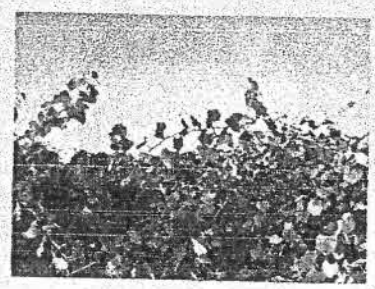

Fig. 1. *Vitis shuttleworthii* grape

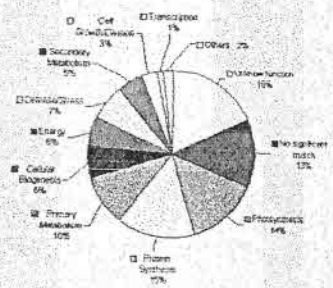

Fig. 2. EST distribution based on protein function using a modified MIPS MATDB *Arabidopsis* Scheme

Table 2. Selective stress and defense relative ESTs found in the *V. Shuttleworthii*

Functional Annotation	Count[a]	Source organism[b]
Defense		
Chalcone synthase	41	*Vitis vinifera*
Chalcone isomerase	27	*Vitis vinifera*
Proline-rich protein	23	*Madicago sativa*
Chitinase	21	*Vitis vinifera*
Pathogenesis-relative protein	19	*Arabidopsis thaliana*
Flavanone 3-hydroxylase	7	*Vitis vinifera*
Stress		
Heat shock protein 70	43	*Arabidopsis thaliana*
Heat shock protein 90	25	*Arabidopsis thaliana*
DnaJ homolog	18	*Salix gilgiana*
Cold induced protein	7	*Solanum tuberosum*
Late embryogenesis abundant protein	4	*Arabidopsis thaliana*
Dehydration induced protein	3	*Arabidopsis thaliana*

[a]Represent Number of ESTs matching the similar gene
[b]Represent the source organism with the most significant BLASTX match

CONCLUSIONS

- An EST database of *Vitis shuttleworthii* grape that is known to be highly disease resistant is being established.
- Preliminary functional annotation indicated that 7% of ESTs were relative to defense and stress proteins.
- There is a high percentage of uniqueness when comparing with existed *V. vinifera* ESTs.
- Over 50 contigs containing complete open coding region of known functional genes were obtained.

FUTURE OBJECTIVES

- Functional annotation of *V. shuttleworthii* EST against other plant genome.
- Identification and isolation of *Vitis* genes responsible for disease resistance and stress tolerance.
- Comparative genomic study between *V. shuttleworthii* and other *Vitis* species including *V. vinifera*.
- Development of SSR and SNP markers from the *V. shuttleworthii* EST data set.

LITERATURE CITED

Michael Bausher *et al.* A expressed sequence tag (EST) set from *Citrus sinensis L.* Osbeck whole seedlings and the implications of further perennial source investigation(2003). *Plant Science* 165(415-422).

Effie Ablett *et al.* Analysis of grape ESTs: global gene expression patterns in leaf and berry(2000). *Plant Science* 159 (87-95).

Molecular characterization of genetic variability and differentiation among European and Asian grape cultivars

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INTRODUCTION

According to Negrul (1938), Asia Minor and the Transcaucasian regions of ancient Eastern civilization is the homeland of cultivated grape (*Vitis vinifera*), where historical data indicate both the beginning of its cultivation as well as the art of wine-making. Wide diversity of ancient varieties closely resembling its wild progenitor ssp. *sylvestris* with small juicy fruit found in these regions are placed in the eco-taxonomic group, *pontica* Negr. Westward advancement of civilization introduced grape cultivation and the art of wine-making into the western lands, where both *in situ* domestication and introgression with introduced grape generated an assortment of wine grapes with small, compact clusters and small juicy fruits closely resembling the ssp. *sylvestris*, which are classified as the eco-taxonomic group, *occidentalis* Negr. Extensions towards the south of Caucasus into Azerbaijan, Turkmenistan, northern Iran where the grape cultivation around the ancient oases in isolation from the wild grape combined with human selection for wine and table types resulted in large number of varieties with typical recessive traits characterized by smooth large leaves, branched clusters with medium-sized juicy fruits which are categorized as *orientalis* Negr.

In the present study we have examined grape cultivars representing these three groups using microsatellite polymorphisms for genetic diversity, differentiation, and relationships.

MATERIALS AND METHODS

One hundred and fifty-nine cultivars representing the three eco-taxonomic groups, *pontica*, *occidentalis*, and *orientalis* along with a fourth group comprising 58 cultivars from Turkmenistan were analyzed for genetic diversity and relationships using 17 polymorphic microsatellite loci. Nei and Li and Nei's unbiased genetic identity were used to generate distance matrices between individuals (binary data) and groups (allele frequencies), respectively. Within group genetic diversity and differentiation between groups were summarized using mean number of alleles, levels of heterozygosity, and Wright's F-statistics. Genetic relationships were elucidated using NJ and UPGMA cluster analyses combined with principal component analysis (PCA).

RESULTS AND DISCUSSION

Extensive polymorphism and high levels of heterozygosity were observed for all the four groups with many unique alleles. The groups appeared to be similar in the mean number of alleles, which ranged from 7.1 for Turkmenistan to 7.5 for the central European group, and the expected levels of heterozygosity ranged from 0.744 for the central European group to 0.717 for Turkmenistan and western European group (Table 1). However, the groups differed significantly for allele frequencies and composition across the 17 loci assayed. The F_{ST}, which is a measure of genetic differentiation among groups accounted for nearly 84% of the total variability with only 18% residing within groups (Table 2).

Table 1. Genetic variability at 17 loci in all populations (SE in parentheses)

GROUP	N	A	H(o)	H(e)
WEST ASIA	26.7	7.2	0.727	0.740
	(0.5)	(0.6)	(0.032)	(0.025)
WESTERN EUROPE	35.3	7.3	0.712	0.717
	(0.8)	(0.7)	(0.046)	(0.041)
CENTRAL EUROPE	34.2	7.5	0.675	0.744
	(1.5)	(0.6)	(0.034)	(0.034)
TURKMENISTAN	36.9	7.1	0.730	0.717
	(1.1)	(0.7)	(0.040)	(0.039)

N = Mean no. of cultivars, A = Mean no. of alleles, H(o)/H(e) = Observed and expected heterozygosity, respectively

Table 2. Summary of F-statistics at all loci

Locus	$F_{(IS)}$	$F_{(IT)}$	$F_{(ST)}$
VVS2	0.010	0.070	0.061
VVMD5	-0.008	0.022	0.030
VVMD7	-0.013	0.039	0.051
VVMD21	0.042	0.069	0.028
VVMD24	0.049	0.092	0.045
VVMD25	-0.055	-0.021	0.032
VVMD27	-0.042	-0.007	0.034
VVMD28	-0.004	0.030	0.033
VVMD29	-0.013	0.023	0.035
VVMD31	0.149	0.223	0.087
VVMD32	0.029	0.066	0.038
VVMD34	-0.095	-0.052	0.039
VVMD36	0.075	0.139	0.069
VRIP62	0.038	0.088	0.052
VRIP79	-0.005	0.046	0.051
VrZAG83	-0.193	-0.158	0.030
VrZAG93	0.145	0.196	0.059
Mean	**0.010**	**0.056**	**0.047**

In the NJ cluster analysis, the western European wine grapes along with a few central European ones formed three distinct groups (Figs 1A and 1B). Several distinct groups containing both the West Asian and Turkmenistan grape with some subgroups exclusively containing Turkmenistan cultivars were evident. However, majority of the central European groups were closely aligned with Asian grape groups.

The distance Wagner tree with Prevosti distance suggested two close affinities, one with West Asian and Turkmenistan cultivars and the other with the European groups (Fig. 2). Although the four groups exhibited overlapping distributions in the PCA, there appeared to be non-overlapping zones containing individuals from distinct groups. However, the central European group was somewhat intermediate found interlocked among the remaining three groups.

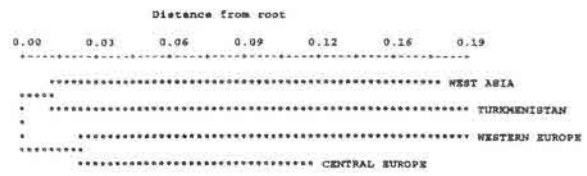
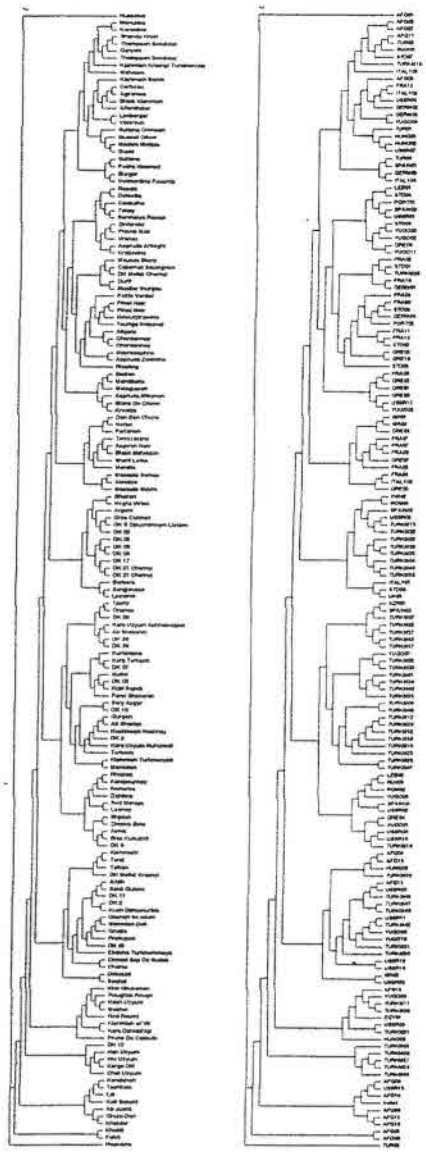


Fig. 2. Genetic relationships among different groups



Figs. 1A & 1B. Genetic relationships among grape cultivars from different groups

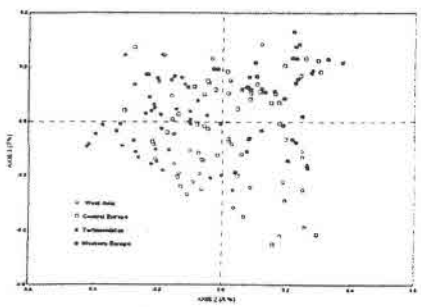


Fig. 2. PCA depicting genetic relationships within and among cultivar groups

CONCLUSIONS

1. Although the level of within group genetic diversity appeared to be similar, the groups differed significantly for frequency and composition of alleles for different loci.
2. There was significant genetic differentiation among groups as suggested by the F_{ST}, which accounted for 84% of total variability.
3. The forgoing results suggest that *in situ* domestication has played a major role along with some level of introgression in the development of grape cultivars in different regions.

REFERENCES

Negrul, A. M. (1938). Evolution of cultivated forms of grapes. *Comptes Rendus (Doklady) de l' Academie des Sciences de l' URSS* 18, 585-588.

» Susan E Gardiner*
» Michael R Cook

HortResearch

A fully automated system to extract DNA from difficult plant tissues

www.hortresearch.co.nz

HortResearch, New Zealand.

Introduction

- Many genomics laboratories now possess the ability to screen DNA in a high throughput fashion. However, the preliminary step of extracting DNA from difficult plant tissues is still mostly performed manually and remains a bottleneck.
- Our plant gene mapping team focuses on the identification of novel genetic markers for various traits in apple, and their use for marker assisted selection of seedlings with durable resistances and improved fruit quality. We have automated PCR reaction set-up and agarose gel-loading using a liquid handling station, but a year ago the initial step of extracting DNA from leaf tissue was still being performed manually at a low throughput.
- Our automated DNA extraction system removes this bottleneck. The most significant component of the system is our custom-designed robot compatible tissue disintegrator. This homogenises freeze-dried tissue samples in a 96 deep well plate format.

Hardware

We have integrated our custom-designed tissue disintegrator into a liquid handling system that performs the DNA extraction. Our device drivers allow seamless operation of both a liquid handler and tissue disintegrator - providing a user-friendly solution to an otherwise laborious task.

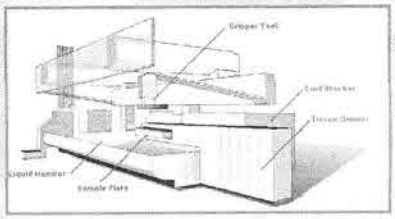
DNA Extraction

We extract DNA using a modified magnetic bead protocol that we are continuing to optimise for plant applications.

Twelve plates each containing 96 freeze-dried plant tissue samples (30 - 70 mg/well) are stored at 4°C on the platform of the liquid handler prior to processing. Each plate is sequentially moved to the grinding work-station, where complete homogenisation occurs in 60 seconds. Homogenisation is uniform across all 96 wells.

The plate is then moved to the DNA extraction section of the liquid handler and processed using the magnetic bead method. The plates are then ready for PCR reaction setup.

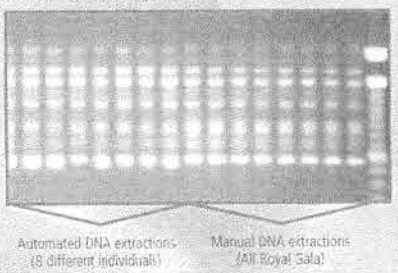
An alternative configuration of the liquid handler with the same overall footprint would allow eight plates to be processed overnight, including PCR and OD analysis in an on-board plate reader.



Results

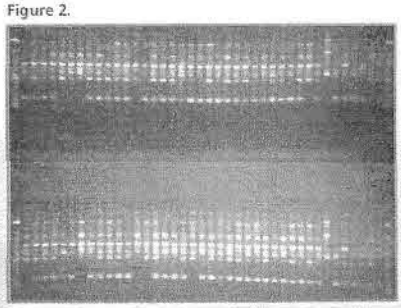
- Extraction of DNA from a 96-well plate of leaf tissue takes approximately 65 minutes, so that DNA from 1152 samples (12 plates) can be extracted autonomously overnight.
- We tested DNA extracted from 8 apple leaf tissue samples using RAPD analysis (as an example of a genetic marker analysis technique requiring high quality DNA). We show the results of product separation by agarosegel electrophoresis, compared with profiles for their manually extracted counterparts (Figure 1).

Figure 1. Automated DNA Extraction Trial: Apple Leaf
All reactions used RAPD primer OPA01



- RAPD profiles from DNA extracted from 80 hops plants demonstrate the consistency of the extractions across the plate - there are no sample failures in Figure 2.

Figure 2.



- We designed our DNA extraction system for use with apple leaves (a "difficult" tissue). We have similar successful results with petunia, clover, ryegrass, tall fescue and maize leaves, as well as pine needles and seeds of ryegrass and clover.
- We have also demonstrated successful microsatellite and SCAR analysis using the automatically extracted DNA.

Discussion

- Our system allows DNA extractions to be performed overnight at the same rate as downstream marker analysis in our laboratory (1152 samples per night). We have effectively increased laboratory throughput by x 90.
- DNA extractions performed by the automated system cost 33% less than manual extractions.
- The high throughput capacity of our extraction system, coupled with cost savings, has enabled us to redesign our mapping experiments, extend our marker assisted selection programmes and will accelerate other genomics studies.

Commercialisation

- Our system will become commercially available early in 2004. Please visit representatives of Alphatech Systems at the MWG-Biotech Booth (404), or contact Alan White on alanw@alphatech.co.nz
- We also perform high throughput DNA extraction and marker screening as a service. Contact: Mike Cook for details on extraction

Microarray Analysis of Defense Against Sclerotinia Stem Rot in Soybean

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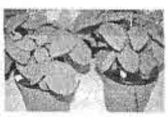
SUMMARY

Sclerotinia stem rot (*Sclerotinia sclerotiorum*), also known as white mold, is a major disease of soybean in the north-central region of the United States. Partial resistance to this pathogen has been reported, yet understanding of the molecular basis of resistance is limited. cDNA microarray technology provides an efficient tool to facilitate searching for genes involved in quantitative traits, such as in defense against Sclerotinia stem rot. The goal of our study was to analyze the profiles of differentially expressed genes in stem tissues of soybean seedlings inoculated with the fungal pathogen at several time points. Two soybean genotypes, Williams 82 (S) and PI194639 (R), were grown hydroponically in a growth chamber under controlled conditions of light intensity and temperature. Cut stem inoculation was performed when soybeans were 2 weeks old. Total RNA samples from stems collected at 0, 3, 6, 18, and 48 h after inoculation were fluorescently labeled with Cy3 or Cy5 dyes and hybridized onto soybean microarrays containing over 9,000 gene representatives. Analysis of variance (ANOVA) assays were performed to identify genes showing significant differential expression. Profiles of PI194639 were compared to that of Williams 82 to identify candidate defense-related genes.

MATERIALS AND METHODS

Plant Materials and Experimental Design

Two repeated experiments
(under controlled conditions)

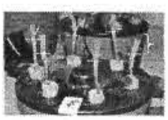


Williams 82 and PI194639
(2-wk old seedlings)

Cut stem technique
(Vuong et al., 2003, Crop Sci.)

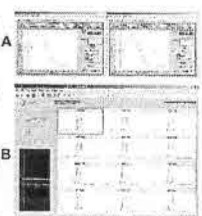
oculated vs. mock inoculated
(*S. sclerotiorum* isolate 105HT)

Stem tissue collection
(after incubation)

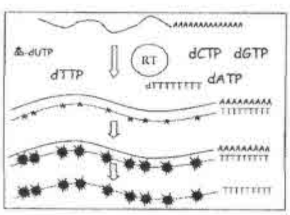


At 0, 1, 3, 6, 18, and 48 hpi
(post inoculation)

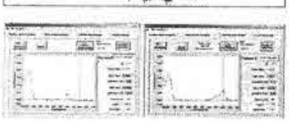
RNA Isolation and Probe Preparation



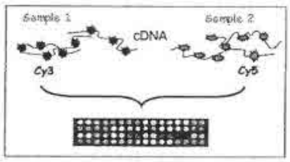
- Extracting total RNA using TRIzol® reagent protocol with minor modifications. (http://www.brinkmann.com/purif_app_PLG9.asp)
- Measuring RNA quantity (Panel A) and quality (Panel B) with both spectrophotometer (NanoDrop) and gel analysis (Aligent 2100 Bioanalyzer).



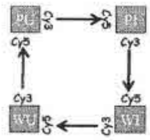
- Performing reverse transcription for cDNA.
- Labeling probes with either Cy3 or Cy5 dye using Amino Allyl Incorporation method.
- Checking quantity of fluorescent labeled probes using spectrophotometer (NanoDrop 1.2)



Hybridization and Scanning

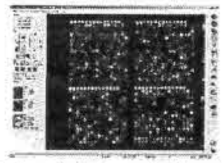


Microarray hybridization



Loop experiment design
P: PI194639; W: Williams 82
I: inoculated; U: uninoculated

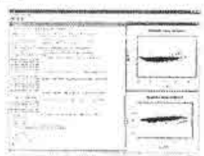
Scanning microarrays (ScanArray™Express)



Computing features and background intensities (GenePix Pro 4.1)

Data Analysis

Gene Traffic
(normalization, spotting, clustering, quality control)



R / MAANOVA
(M-A plot, t-test, F-test)

RESULTS

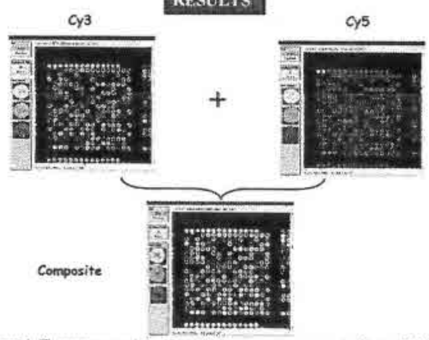


Figure 1: Fluorescence emission from a microarray was converted into a digital output for each dye. Image analysis software was used for quantification of individual array elements, and median pixel intensities for each element were calculated.

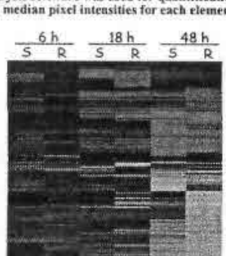


Figure 2: Hierarchical clustering of genes showing at least 3X differential expression at 6, 18, and 48 hpi.

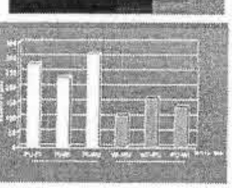


Figure 3: Number of differentially expressed genes selected at the probability value *P<0.01* at the point 6 hpi.

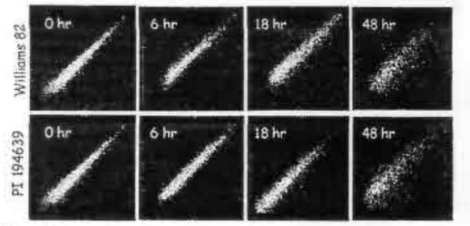


Figure 4: Scatter plots showing differential expression of genes in PI194639 and Williams 82 between inoculated and mock at different time points. T-O plc is Williams vs PI194639 prior to inoculation to show basal differences.

Table 1: Genes showing significantly (*P<0.01*) differential expression in PI194639 inoculated with the pathogen *S. sclerotiorum*.

No	Gene description	P-value		
		PU-PI	PI-WI	PI-WU
1	No hits found	0.001902	0.002602	0.004097
2	[illegible]	0.001279	0.004524	0.001144
3	[illegible]	0.008565	0.003322	0.010011
4	carbonic anhydrase [illegible]	0.005409	0.008171	0.002216
5	[illegible]	0.002142	0.000556	0.002074
6	[illegible]	0.004048	0.001204	0.001846
7	chlorophyll a/b-binding protein type I precursor [illegible]	0.005077	0.005852	0.002094
8	[illegible]	0.007399	0.007327	0.002536
9	[illegible]	0.008659	0.007725	0.004171
10	putative protein [illegible]	0.00507	0.006971	0.00617
11	[illegible]	0.004174	0.009307	0.000705
12	[illegible]	0.002721	0.006112	0.002146
13	RSTA_ORYSHI 60S RIBOSOMAL PROTEIN [illegible]	0.002765	0.000491	0.004772
14	glutathione S-transferase GST 8 [illegible]	0.00455	0.008956	0.000042
15	ATP-dependent [illegible]	0.009371	0.009206	0.007006
16	[illegible]-binding transcription activator [illegible]	0.000424	0.000908	0.000267

FUTURE WORK

- Finish hybridizing to arrays with additional replications and different time points.
- Complete statistical analyses to identify genes associated with defense to white mold in PI194639.
- Use sequence information to develop molecular markers of genes of interest. Genes that show strong correlation with resistance will be mapped (Fig. 5, Panel A and B below).
- Putative defense-related genes will be further analyzed to verify their functional role in resistance.

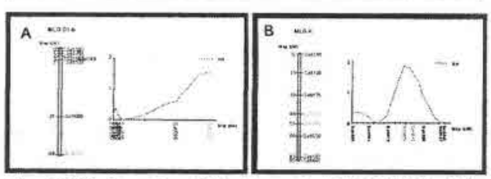


Figure 5 : QTLs for resistance to Sclerotinia were identified in MLG D1-b and K. Several SSR markers showed significantly associated with the QTLs.

CITED REFERENCES

1. Kerr, M.K. and G.A. Churchill. 2001. Experimental design for gene expression microarrays. Biostatistics 2:183-201.
2. Wu, H., Kerr, M.K., Cui, X., and Churchill, G.A. 2003. MAANOVA: A Software Package for the Analysis of Spotted cDNA Microarray Experiments. *In* The Analysis of Gene Expression Data : Methods and Software. Springer. pp. 313-341.
3. Vuong,T.D., D.D. Hoffman, B.W. Diers, J.K. Miller, J.R. Steadman, and G.L. Hartman. 2003. Utilization of the cut stem inoculation method to evaluate soybean, dry bean, and sunflower for resistance to *Sclerotinia sclerotiorum*. Crop Sci. (in press).

ACKNOWLEDEGEMTS

The authors wish to thank the **Sclerotinia Initiative**, USDA-ARS, for providing funds for this research project, J. C. Nicholas and students for technical assistance.

GOBIERNO DE CHILE
FUNDACIÓN PARA LA INNOVACIÓN AGRARIA

10. Aspectos Administrativos

10.1. Organización previa a la actividad de formación

a. Apoyo de la Entidad a cargo de la organización del viaje

X bueno ____ regular ____ malo

(Justificar)

b. Información recibida durante la actividad de formación

_X_ amplia y detallada ____ aceptable ____ deficiente

c. Trámites de viaje (visa, pasajes, otros)

_X_ bueno ____ regular ____ malo

d. Recomendaciones (señalar aquellas recomendaciones que puedan aportar a mejorar los aspectos administrativos antes indicados).
Recomendaría simplificar la rendición económica y entregar viáticos generales de movilización y alimentación que no se rindan con boletas y detalles. No siempre se cuenta con este tipo de recibos en las organizaciones de Congresos, además que se evita el estrés del participante de mantener todo este material hasta la vuelta del evento. Sólo aquellos items de mayor envergadura, tales como inscripción, hotel, u otro tipo de adquisiciones debieran ser respaldados por documentos.

11. Conclusiones Finales: entregar las conclusiones finales del participante de la actividad de formación, incluyendo el nivel de satisfacción de los objetivos personales.

En general la actividad de formación cumplió con las expectativas y objetivos planteados. No siempre se tiene acceso a Conferencias de esta envergadura, dónde se presentan los grupos líderes de investigación científica. El apoyo de FIA para la asistencia a este evento significó un apoyo invaluable a las iniciativas Genoma Chile, ya que permitió a cuatro (inicialmente cinco) de sus miembros asistir a este Congreso. El conocer el desarrollo de sus proyectos, estrategias y limitaciones permitirá fortalecer el trabajo que aquí se realice, al incorporar sus experiencias. Sin duda, también es una gran oportunidad de conectarse con el mundo científico internacional, además de difundir las actividades que en Chile se realizan.

Fecha: 9/02/04

Nombre y Firma beneficiario de la beca: Inés Marlene Rosales V.