

KS-1

Advances in Tissue Engineering. ROBERT LANGER. Massachusetts Institute of Technology, 45 Carleton Street, Room E25-342, Cambridge, MA 02139. Email: rlanger@mit.edu

Approaches involving the synthesis and application of bioerodible polymers to serve as implantable scaffolds for mammalian cells to create new tissues and organs are being studied. This talk will discuss the design of new materials in particular synthetic polymers with specific ligands attached to them, photopolymerized materials, shape memory degradable polymers, rubbery polymers and materials with reversibly switching surfaces that may have applications in these areas. We will also examine the use of materials coupled with human embryonic stem cells or other cells, and the application of these approaches to the creation of new tissues. This approach has been used to create a variety of tissues such as liver, skin, nerves, blood vessels, cartilage, heart muscle, and other tissues in animals and humans.

PS-1

Interplay of Gene-specific Disease Resistance, Basal Defense, and the Suppression of Host-responses. R. A. Caldo¹, D. Nettleton², and R. P. WISE^{1,3}. ¹Department of Plant Pathology and Center for Plant Responses to Environmental Stresses; ²Department of Statistics; and ³Corn Insects and Crop Genetics Research, USDA-ARS, Iowa State University, Ames, IA 50011-1020. Email: rpwise@iastate.edu

Active plant defense to microbial attack is highly dependent upon recognition events involving associated gene products in the host and the pathogen. Both perception of general and specific pathogen-associated molecules result in signal transduction cascades ultimately leading to disease resistance. To ascertain the global framework of host gene expression during biotrophic pathogen invasion, we have utilized the 22K Barley1 GeneChip to analyze the transcriptional regulation of 22,792 host genes throughout various interactions among barley and the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*). Near-isogenic barley lines with introgressed *Mla* CC-NBS-LRR resistance alleles and associated mutants were utilized to identify genes with significant differential expression in incompatible and compatible interactions. Using linear mixed model analyses, over 50 genes exhibited highly similar patterns of up-regulation among all incompatible and compatible interactions up to 16 hours after inoculation (hai), coinciding with germination of *Bgh* conidiospores and formation of appressoria. By contrast, significant divergent expression was observed from 16 to 32 hai, during membrane-to-membrane contact between fungal haustoria and host epidermal cells, with notable suppression of most transcripts identified as differentially expressed in compatible interactions. Based on these results, we propose a model that links the recognition of general elicitors and specific avirulence proteins in the expression of plant defense responses, supporting the hypothesis that host-specific resistance evolved from the recognition and prevention of the pathogen's suppression of plant basal defense.

PS-2

New Markers for Human Tumors: A High-throughput Computational Approach. A. BARANOVA, L. Krukovskaya, T. Tiazhelova, and A. P. Kozlov. ¹George Mason University, Fairfax, VA 22030 and ²Biomedical Center, St. Peterbourg, RUSSIA. Email: abaranov@gmu.edu

Tumor markers are molecules that indicate the presence of malignancy. Tumor markers and corresponding antibodies that are present in serum could be useful for early diagnostics of primary tumors and relapsed disease, as well as for determining tumor prognosis and predicting likely response of the tumor to therapy. Tumor markers are part of the clinical routine. Nevertheless, lack of sensitivity and specificity precludes routine usage of single tumor markers in population-based screening. Shortcomings of single tumor markers could be solved by parallel evaluation of multiple tumor markers that can produce required certainty. Genome and proteome-wide approaches currently lead to identification and initial characterization of hundreds new tumor marker candidates. Most prominent of such methods are serological analysis of recombinant cDNA expression libraries (SEREX), 2-dimensional polyacrylamide gel electrophoresis, mass spectrometry, as well as protein and DNA microarrays. Last but not least is a computational approach allowing high-throughput detection of tumor marker candidate genes in publicly available data sets. Tumor marker encoding genes possess characteristics that make them different from typical human genes. Many genes with these characteristics may be active in tumor cells but not in any normal cell type of the organism. The prediction concerning the activation of many sequences in tumors was open to experimental verification. Using the computational differential display, we have been able to identify a considerable amount of human tumor-specific EST clusters, many of which do not contain long open reading frames. A computer-based differential display tool named HsAnalyst has been developed and successfully used for comparison of expression patterns in a set of tumors versus a set of normal tissues. All available tumor libraries were subtracted against all available normal libraries instead of pairwise comparisons of each tumor and corresponding normal tissue. A list of EST clusters highly represented in tumors and rarely observed in normal tissues has been developed as a resulting output file of the program. Revealed tumor-specific sequences can indeed serve as new tumor markers, as we showed experimentally that many of them showed very restricted tissue expression pattern or entirely tumor-specific pattern. Most of these sequences are evolutionary new, as they are absent in rodent genomes and/or located in the recently duplicated chromosomal regions.

PS-3

Plant-made Pharmaceuticals: Where Are We Now? D. R. BETHELL. Ventria Bioscience, Sacramento, CA 95834. Email: dbethell@ventria.com

Production of pharmaceuticals in plants is an age old technique - the discovery of digitalis in foxglove of the English garden is an early example. Traditional plant breeding techniques have been used to improve plant production of these pharmaceuticals. Today new scientific tools have made further advances possible and plants can produce new proteins for the improvement of human health. Plants can be engineered to produce proteins as well as peptides. Examples include complex proteins (monoclonal antibodies, human serum albumin), breast-milk proteins (lactoferrin, lysozyme) and peptides (growth hormone, parathyroid hormone, IGF). Plants are easily scalable and economically superior with a fraction of the capital cost. Work on plant-made pharmaceuticals (PMP) has been done in alfalfa, corn, lemna, rice, barley, safflower and tobacco with the promise of treating cancer, inflammatory bowel disease, obesity, rheumatoid arthritis, cystic fibrosis and pediatric and geriatric diarrhea. Proteins produced in plants also provide a non-animal source of growth factors and additives for use in cell culture systems, avoiding the issues of viral and prion contaminations. The first of the PMP products are beginning to move through human clinical trials and the regulatory system. An update on the progress and successes in the PMP arena will be reviewed.

PS-4

Directed Tissue Self-assembly. GABOR FORGACS. George H. Vineyard Professor of Biological Physics, University of Missouri-Columbia, Department of Physics & Biology, Columbia, MO 65211. Email: forgacsg@missouri.edu

Morphogenesis is under strict genetic control. However, genes do not create forms, physical mechanisms do. Based on this recognition, in order to dissect principles of biological self-assembly and the interplay between controlling molecular and biophysical factors, we considered several specific shape forming processes, both experimentally and through modeling. In particular, we studied the fusion of cardiac cushions. The biophysical framework that we relied on was tissue liquidity, a notion, according to which embryonic tissues mimic classical liquids. We found that cardiac cushions fuse, both in vivo and in vitro, as liquid drops. The striking analogy between real and model patterns provided new insight into early morphogenesis. These patterns demonstrated the crucial role of the synergy between cell-cell and cell-matrix interactions in the development of early forms. We will show, based on our findings, how novel tissue engineering technologies could be employed to build living structures, such as organ modules, under laboratory conditions.

PS-5

Engineering Connective Tissue Grafts via Antigen Reduction Technologies. STEVEN GOLDSTEIN, Patti Dawson, Steve Walsh, and Albert Heacox. CryoLife, Inc., 1655 Roberts Boulevard, NW, Kennesaw, GA 30144. Email: goldstein.steven@cryolife.com

Broadly conceived, tissue engineering encompasses multiple technologies directed toward providing implantable constructs to replace damaged or diseased natural structures; these replacements are expected to exhibit normal function and display certain metabolic activities. The means to achieve this goal range from total synthesis of the replacement to modification of the properties of donor structures to enhance their post-implantation activity. A platform technology (SynerGraft®) has been developed for the two-fold purpose of reducing the antigenicity of natural connective tissue structures and enhancing their biocompatibility once implanted. As evidence of a novel scaffolding function in the treated tissues, re-cellularization of these matrices has been detected and remodeling by the action of host cells indicate an interstitial cell phenotype. SynerGraft treated bovine ureters are presently used to create arteriovenous-bridges in dialysis patients with failed native fistulae and/or synthetic grafts. Many of the grafts have been in continuous use for over 900 days with no evidence of rejection of the non-crosslinked xenograft tissue. As is typical of tissue grafts, infection incidents are few even with frequent graft access. Additionally, the implantation of similarly decellularized human heart valves has significantly minimized the allosensitization typically demonstrated in allograft valve recipients. Antigen reduction technology has been used to successfully modify the behavior of connective tissues, enabling cross species implants and directing use of structures beyond their natural function.

PS-6

Industrialized Metabolite Profiling: An Effective and Efficient Tool for Discovery in the Life Sciences. R. TRETHERWEY. Metanomics GmbH & Co KGaA, Tegeler Weg 33, 10589 Berlin, GERMANY. Email: company.info@metanomics.de

Metanomics has pioneered the use of industrialized metabolite profiling as a discovery tool in the life sciences. Considerable effort has been focused on providing the foundations for novel metabolic engineering strategies in crop plants. Given that such work is both lengthy and complex it is important to be as precise as possible in the selection of the lead genes that are taken into development. metanomics has run large-scale genomics programs with the aim of filtering out key genes that serve as candidates for the engineering of metabolism or physiological responses in crop plants. The creation of genetic diversity on a genome scale in Arabidopsis has been coupled to broad-based, high throughput metabolite profiling, phenotypical and physiological screening. The scale of the data sets generated in this work has necessitated the pioneering of new informatics systems for process control, data validation and data mining. In this presentation, the results of these programs will be introduced and the case made that they are effective and efficient in linking genes to particular metabolic and physiological functions such as stress tolerance. Further, the results from this work illustrate the broad sensitivity of the metabolic network to changes in genotype or environment. It will be argued, with examples from animal toxicology and human health that this conclusion is of general importance in the life sciences.

PS-7

The Use of Animal Cell-based Toxicity Sensors for Water Testing. W. H. VAN DER SCHALIE¹, T. P. Gargan, II², T. R. Shedd¹, and M. W. Widder¹. ¹U.S. Army Center for Environmental Health Research, 568 Doughten Drive, Fort Detrick, MD 21702-5010 and ²Geo-Centers, Inc., U.S. Army Center for Environmental Health Research, 568 Doughten Drive, Fort Detrick, MD 21702-5010. Email: william.vanderschalie@det.amedd.army.mil

Deployed soldiers may encounter contamination of drinking water sources from a wide range of industrial and agricultural chemicals. Since a rapid, comprehensive analysis for both organic and inorganic toxicants is impractical in many situations; an alternative is to use biologically-based toxicity sensors capable of responding to many different chemicals. Available sensors utilize enzyme systems, bacteria or animal cells. Examples of animal cell systems (and endpoints) include neuronal micro-electrode arrays (spike rate of neurons), fish chromatophores (pigmentation dispersion), bovine pulmonary artery endothelial cells (electrical impedance) and human liver cells (low density lipoprotein uptake). The ability of a set of biologically-based toxicity sensors to respond rapidly and at appropriate levels of sensitivity to chemicals with varying modes of toxic action is being evaluated through blind sample testing by several laboratories. Results will be used to identify which sensor (or combination of sensors) may be suitable for water testing. Besides toxicant sensitivity, issues for using animal cells in toxicity sensor systems include maintaining cell viability during transportation and storage, biological response variability, and water sample preparations prior to testing. Possible Army applications for a cell-based toxicity sensor include use at field water production sites as well as at fixed facilities such as water treatment plants. Domestic water utilities would benefit as well from improved capabilities to rapidly identify toxic conditions in source or product waters.

PS-8

Use of Invertebrate Cells as Biosensors. G. SMAGGHE. Lab Agrozoology, Ghent University, Ghent, BELGIUM. Email: guy.smagge@ugent.be

At present, many international research groups are testing different chemistries using invertebrates in order to gain basic understanding in their ecotoxicology. In this review attention goes to different types of compounds including pesticides with classical neurotoxins and more modern insecticides with an insect growth- and development-disturbing action, and endocrine disrupting chemicals (EDCs) including natural chemicals and pharmaceuticals. In this paper some relevant examples to address some key questions are discussed: e.g. which invertebrates are likely to be vulnerable to mammalian and non-mammalian toxicity and hormone-disturbing, which types of invertebrate chronic tests might be most sensitive and cost-effective to address potential environmental exposures? Where do we stand in our progress on the understanding of EDCs and appropriate extrapolation of invertebrate chronic toxicity data to the wider world, and what do we understand so far of their mode of action using *in vitro* steroid hormone receptor-screening assays?

A-1

Electroporation is a Versatile Tool for Delivering Plasmid DNA. R. HELLER. Department of Medical Microbiology and Immunology, College of Medicine, Center for Molecular Delivery, University of South Florida, Tampa, FL 33612-4799. Email: rheller@hsc.usf.edu

A key issue in the development of effective gene transfer protocols is the development of appropriate delivery methods. Typically, the goal is to target gene delivery to a particular type of cell or to cells within a specific tissue. The delivery of genes that code for biologically active compounds is envisioned as a treatment for many diseases. The uptake of molecules through the cell membrane can be facilitated by electroporation, a physical phenomena that temporarily permeabilizes cell membranes. When membranes are in a permeabilized state it is possible for molecules that do not normally pass through the membrane to gain intracellular access. Electroporation can be used for drug or plasmid DNA delivery either alone or in combination. Sets of electroporation parameters, which includes electric field strength, pulse duration, number of pulses, electrode geometry and configuration, can be chosen to deliver plasmid DNA in such a way as to manipulate the onset, level, and duration of protein expression. One area that has shown a great deal of promise is the treatment of solid tumors. Long-term complete regressions of established tumors have been obtained with a plasmid encoding for IL-12 in a murine melanoma model. Recently a Phase I clinical trial was initiated using this approach. The potential for this delivery approach is just now being realized.

A-2

In Vivo Gene Delivery with Pulsed Electrical Fields. R. W. MALONE. Gene Delivery Alliance, Inc., Jefferson, MD 21755. Email: gtinventor@hotmail.com

Non-viral gene delivery research currently confronts three significant obstacles, each of which contribute to the modest and transient levels of transgene expression that are often observed; 1) Exogenous polynucleotides are rapidly degraded by cytoplasmic and extracellular nucleases, 2) when used *in vivo*, most non-viral gene delivery technologies are very inefficient for delivering the payload into cytoplasm, and 3) peak transgene expression is typically observed in the first few days and then rapidly declines (often to undetectable levels). Results obtained from studies addressing each of these issues will be reviewed, with a particular focus on unpublished work involving electroporative lung transfection and transplantation in the rat model. The results to be presented demonstrate that DNA degradation and clearance may be reduced by formulation with a broad-spectrum nuclease inhibitor, electroporation may be safely used *in vivo* to efficiently permeabilize cells enabling plasmid uptake directly into cytoplasm, and an efficient housekeeping promoter known to provide prolonged transgene expression in lung can yield sustained transgene expression after electroporative delivery. Lung electroporation conditions were optimized at four 300V/cm, 20ms pulses administered at 1Hz by flat electrode plates. After transplanting lungs transfected with a human ubiquitin promoter driving luciferase expression, at least 40 days of high-level transgene expression were observed. By combining these factors with optimized DNA/ATA formulations, the data indicates that levels of transgene expression in transfected lung tissue may be increased by 15,000 fold relative to "naked DNA".

A-3

Transfection of Neurons and Neural Stem Cells by Nucleofection. E. L. K. GOH, D. Ma, and H.-J. Song. Institute for Cell Engineering, Department of Neurology, Johns Hopkins University, School of Medicine, Baltimore, MD 21205. Email: egoh@jhmi.edu

The ability of stem cells to differentiate into a variety of cell types renders them attractive for basic research as well as therapeutic applications. The transfection of stem cells is the main limiting factor for the progress in the genetic engineering of such cells during the past decade. In order to elucidate the molecular mechanisms of proliferation, differentiation and self renewal properties of neural stem cells, the availability of an efficient, straight-forward transfection of stem cells for genetic manipulation purposes is of tremendous importance. Similarly, primary cells that are able to provide an *in vitro* system with more physiological relevance as compared to cell lines, are also hard to transfect. They are normally very sensitive and vary significantly in the conditions needed to achieve efficient transfection results. Many methods reliably working for cell lines simply fail with primary cells. Especially cells displaying slow or even no growth such as neurons are hard to transfect, since the nuclear membrane remains intact prohibiting efficient gene transfer to the nucleus for transcription. Nucleofection^(*) has recently been shown to address the problems in transfecting cells such as primary and stem cells. Latest data obtained with transfected primary neurons and neural stem cells will be presented and their impact on research, development, and therapy discussed

A-4

Genetic Dissection of Human Papillomavirus Oncogene Functions in Organotypic Epithelial Raft Cultures. THOMAS R. BROKER and Louise T. Chow. Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294-0005. Email: broker@uab.edu

Human papillomavirus (HPV) establishes infections in the cycling basal keratinocytes of squamous epithelia. The viral DNA persists as extrachromosomal plasmids that replicate when these cells enter S phase periodically. Vegetative amplification of the viral genome takes place in squamous epithelium undergoing terminal differentiation. The productive program of HPV infection has been recapitulated in organotypic raft cultures of human keratinocytes grown at the medium:air interface. Viral genes can be introduced into the keratinocytes by retrovirus-mediated transduction. Under the control of their native promoter, expression is differentiation-dependent, as in natural lesions. The E7 protein of both high-risk HPV-18 and low-risk HPV-11 genotypes interacts with the host retinoblastoma susceptibility protein (pRB) and reactivates S phase in a subset of post-mitotic, differentiated keratinocytes in raft cultures. S phase reentry depends on E7 binding to pRB, and residues in HPV-11 E7 critical for efficient promotion of S phase have been identified. In a separate subset of differentiated keratinocytes, E7 also provokes an antiviral defense in the form of p21cip1/cyclin E costabilization. This latter response is initiated by the constitutive expression of p27kip1 in many of the differentiated cells. Stable, inactive complexes are formed between p27kip1 or p21cip1 and cyclin E/cdk2, a kinase necessary for viral DNA replication. These novel virus-host interactions are also observed in patient specimens. The role of cyclin E/cdk2 in modulating the shuttling of the viral DNA helicase E1 between cytoplasm and nucleus and in overcoming the normal licensing of replication origins to achieve vegetative amplification will be discussed.

A-5

Using the HBV Recombinant Baculovirus/HepG2 System to Understand HBV Pathogenesis. H. C. ISOM. Department of Microbiology and Immunology, Penn State College of Medicine, Hershey, PA 17033. Email: hci1@psu.edu

Worldwide, 350 million individuals are chronically infected with hepatitis B virus (HBV). Approximately one of every four HBV carriers will eventually succumb to chronic active hepatitis, cirrhosis, or hepatocellular carcinoma (HCC). Although effective vaccines exist, vaccination is expensive, not readily available in all parts of the world and not all individuals develop immunity after vaccination. The currently approved treatments for HBV are interferon α , lamivudine, and adefovir. A problem with these treatments is that upon cessation of therapy, HBV DNA rapidly reappears in patient sera. This rebound is assumed to be due to the persistence of an intermediate in the HBV life cycle, HBV covalently closed circular (CCC) DNA, present in the nucleus of infected hepatocytes. Therefore, it is necessary to devise novel therapies that function to block multiple different steps in the HBV life cycle and to have a reproducible *in vitro* system in which the effects of these therapies on HBV CCC DNA and the rebound phenomenon can be measured. Our laboratory has generated an HBV recombinant baculovirus which delivers replication competent HBV DNA to human HepG2 cells with high efficiency. HBV gene expression is driven exclusively from endogenous promoters. Levels of HBV replication can be achieved that are markedly higher than from conventional HBV-expressing cell lines. All forms of HBV DNA including CCC DNA can be readily detected by Southern blot analysis. Replication reaches a plateau level by 5-6 days p.i. and remains at that level for 30 days, making it possible to use this system to systematically observe the effects of antivirals on CCC DNA and to follow rebound after release from antiviral treatment. HBV is an etiologic agent for HCC. Evaluation of novel antiviral strategies that reduce or eliminate HBV CCC DNA is highly critical for reducing HCC incidence.

A-6

Methylthiophanate-induced Genotoxicity and Development of Single Strand Breaks in DNA. J. MUSARRAT and Q. Saquib. Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh 20 202, U.P., INDIA. Email: musarratj1@yahoo.com

The toxicity data of methylthiophanate [MT] (dimethyl 4,4'-*o*-phenylenebis [3-thioallophanate]) indicate the associated hazards of this systemic fungicide on both the target and non-target organisms. However, its genotoxicity and extent of DNA damage have not been critically pursued from structure-activity relationship stand point. This has prompted us to study its direct DNA damaging potential. The genotoxicity was assessed using DNA repair defective *uvr A*, *rec A*, *lex A*, *pol A* mutants of *E. coli* K12. Cells treated with varying concentrations (0-600 μ M) of MT exhibited significant reduction in the colony forming ability *vis-à-vis* wild-type AB 1157. Maximum decrease (82%) in the survival was noticed with *pol A* mutant followed by *lex A*, *rec A* and *uvr A* mutants as compared to their wild-type counterpart, upon 6 h exposure with 600 μ M MT at 37° C. Significant damage in the DNA polymerase-I defective cells suggests the formation of MT-induced strand breaks in cellular DNA. The nature and extent of DNA strand breaks generated were determined using alkaline single cell gel electrophoresis, SCGE (comet assay) of MT-treated human lymphocytes in RPMI medium at 37° C. The SCGE data revealed MT dose-dependent DNA migration from cell nuclei. At the highest concentration of 1000 μ M MT, the DNA damage parameters were measured as Olive tail moment (40.3 AU), Tail DNA (44.4%) and Tail Length (203.2 μ m) ($p < 0.001$) as compared to insignificant DNA migration in untreated control, which validated the *ex-vivo* development of single strand breaks (ssb) in treated DNA. The number of ssb quantitated *in vitro* as 1.5 breaks per unit calf thymus DNA treated with 1000 μ M MT following the alkaline unwinding elution assay. In view of the significant genotoxicity and strand breaks formation, a plausible mechanism of MT-DNA interaction has been deduced. The results unequivocally suggests MT as a genotoxic fungicide with a potential of triggering carcinogenesis, in case the damage is not accurately repaired by the cellular DNA repair machinery. (This work was supported by CSIR Grant # 37(1124)/03/EMR-II and MAAS Grant # MAAS/RS/01, sanctioned to JM).

A-7

Authentication and Characterization of Animal Cell Lines at the ATCC. YVONNE A. REID. ATCC, 10801 University Blvd., Manassas, VA 20110. Email: yreid@atcc.org

Animal cell culture technique is widely used by scientists in many diverse disciplines such as cell biology and genetics for transcriptome and proteome analyses. As a result, cell line authentication and characterization are central activities for the ATCC. Nevertheless, these critical activities are the most under appreciated responsibilities facing all Biological Resource Centers. Recent advances in new technologies have led to improved and more accurate description of cell lines and as a result many cell lines have been shown to be misidentified. Recently, two cell lines RF-1 (CRL-1864) and RF-48 (CRL-1863) which were submitted to ATCC in 1989 and reported to be B cells isolated from a lymphoma were found by Ji J. et al. (Oncogene, 2002) to be of gastric origin using microarray analysis. These results were confirmed by the ATCC using FLOW cytometry. The financial loss as well as the scientific setback to these types of errors is estimated to be millions of dollars. Therefore, accurate cell line authentication and characterization require a comprehensive strategy that employs a battery of multiple complementary technologies. This includes: test for microbial contaminants; tests for cellular cross contamination and or misidentification; conformation of tissue type using tissue specific markers as well as tests for gene and protein expressions. An overview of the current technologies used by the ATCC to characterize animal cell lines will be presented.

A-8

Signs of Pluripotency: Characterization of Embryonic Stem Cell Lines. RICHARD JOSEPHSON. Stem Cell Center, American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110. Email: rjosephson@atcc.org

Human embryonic stem cells (hESC) offer a renewable source of a wide range of cell types for use in research and cell-based therapies for disease. The characterization of these cells provides important information about the current state of the cells and affords relevant details for subsequent downstream manipulations. This presentation will discuss current methods of hESC characterization, including not only common markers of the undifferentiated state, but also pluripotent *in vitro* differentiation and quality control. Immunocytochemical markers of undifferentiated and differentiated hESC are assayed by immunofluorescence and flow cytometry. Flow sorting is also used to purify undifferentiated hESC for subsequent gene expression analysis. Assays of quality include sterility, identity, clonality and chromosomal integrity. STR and HLA typing allow confirmation of cell line identity and offer a sensitive measure of contamination by other lines. Karyotypic instability is common in hESC (Draper, J. et al., 2004) and problematic for cell replacement therapy. Thus frequent karyotyping is a crucial element of characterization. In addition, we will discuss the ongoing search for new and more informative markers of pluripotency and genetic stability in ESCs.

A-9

DNA Profiling and the Authentication of Cell Lines. JOHN R. W. MASTERS. University College London, 67 Riding House Street, London W1W 7EJ, UNITED KINGDOM. Email: J.Masters@ucl.ac.uk

DNA profiling uses short tandem repeats (STRs), which are highly variable between individuals, for testing paternity and crime suspects. STRs are short sequences of repetitive DNA, normally 2-5 base pairs in length, that are repeated many times. For example, the 20 base pair sequence tgcgatgcatgcatgca is 5 copies of the STR tgca. The polymorphisms in STRs are due to the different number of copies of the repeat element that can occur in a population of individuals. By analysing enough STRs (approximately 10), DNA profiling has the statistical power to distinguish between every individual worldwide (except monozygotic twins). When developing a new cell line, DNA profiling is invaluable for the purpose of authentication. DNA from a tissue or blood sample should be extracted and stored at the same time as the primary culture, and if a cell line is established from, either normal or cancer tissue, the origin can be unequivocally confirmed by comparing the DNA profiles. By undertaking DNA profiling at the time the cell line is established, an unambiguous method is available for the authentication of that cell line indefinitely, from whatever laboratory or other source the cells are obtained. In contrast to earlier methods of cell line authentication, DNA profiling is relatively simple to reproduce between laboratories and is inexpensive (\$100-200). DNA profiling is needed to exclude cross-contamination, which is now widespread. For example, the first continuous cell line derived from a human cancer, HeLa, was developed in 1952 from a glandular cancer of the cervix. In 1967, using isozyme analysis, it was shown that most of the human continuous cell lines developed since 1952 were HeLa cells, indicating cross-contamination. These false cell lines include KB, HEP-2, Int407, Chang liver and WISH cells. Despite many publications showing that these cell lines are indistinguishable from HeLa cells genetically, they continue to be used respectively as models of skin and head and neck cancer, fetal intestine, hepatocytes and amniotic cells. Similarly, the ECV304 cell line is used as a model of normal endothelium, despite the fact that it has been shown to be the T24 bladder cancer cell line. The cross-contaminating cell line is frequently claimed to show characteristics of the tissue from which it was thought to be derived. Since there have been a large number of publications demonstrating the false nature of these cross-contaminated cell lines, there seems little reason for their continued use, unless it is made clear that the model system being used is derived from HeLa or one of the many other cross-contaminating cell lines.

A-10

High Passage Number of Continuous Cell Line Affects the Transcriptome. S. Finn¹, J. Han², R. Farnsworth¹, Amy Yang², Raj Puri², and P. IKONOMI. Molecular Authentication Resource Center, American Type Culture Collection, Manassas, VA 20110 and ²Center for Biologics Evaluations and Research, Bethesda, MD. Email: pikonomi@atcc.org

The rapid development in genomic and proteomic analysis has increased the need for the standardization of cell culture conditions and protocols in order to compare mRNA expression levels among different cell cultures. The accepted practice is to compare data among laboratories using cell lines that may be at significantly different passage numbers, but does not take into account potential changes in the cell phenotype. Several recent publications have reported that cells do undergo significant changes in phenotype with increased passage number. In order to address the phenotypic drift in continuous cell lines with increased passage number, we have conducted comparative analyses of six different cell lines, CaCO-2 (ATCC.HTB-37), IMR-32 (ATCC.CCL-127), LNCap.FGC (ATCC.CRL-1740), MCF-7 (ATCC.HTB-22), WI-38 (ATCC.CCL-75), and THP-1 (ATCC.TIB-202) obtained from ATCC and another distribution source. These cell lines are of various tissue origin and the difference in passage number ranges from 12 to 76. We assessed the passage number, cell growth rates, and examined gene expression profiles by microarray analyses. Significant changes in growth rate were observed which correlated with the difference in the number of cell passages. The microarray analyses revealed significant variation in gene expression among cell lines of low passage numbers compared cell lines at higher passage numbers. While no significant differences were observed between two samples at low passage numbers. These differences were then confirmed by quantitative PCR analyses using several target genes and the results demonstrate that increased passage numbers influence both growth rate and gene expression profiles in cell lines. Additionally, this information underscores the importance of using low passage cell lines in order to provide accurate and reliable gene expression information.

A-11

Stem Cell Regulation in Japan. RYUICHI IDA. Kyoto University, Faculty of Law, Graduate School of Law, Former Chairperson, International Bioethics Committee, UNESCO, Former Member, Expert Panel on Bioethics, Council for Science and Technology Policy, Cabinet Office, JAPAN. Email: ida.ryuichi@law.kyoto-u.ac.jp

Japan has enacted the Clone Technology Act in April 2000. This act, on the one hand, prohibits the transplantation of somatic cell nuclear transferred embryo, or so called "cloned human embryo" into the uterus (ban on human reproductive cloning), and, on the other hand, leaves the therapeutic cloning under the control of the MEXT (the Ministry of Education, Culture, Sports, Science and Technology), which have set a moratorium on therapeutic cloning until the status of human embryo would have been clarified. Meanwhile, the Guidelines for Derivation and Use of Human ES cells were established by the MEXT in September 2001. Research both on derivation and on the use of human ES cells is thus permitted under strict conditions, inter alia, obtaining the informed consent and limitation to ES cells only from supernumerary embryos, and with national ethical review system. In July 2004, the Council of Science and Technology Policy (CSTP), following the conclusion of its Expert Panel on Bioethics (which may be deemed as Japanese National Bioethics Committee), decided to remove the moratorium on therapeutic cloning, waiting for new guidelines on this matter (expected for the next year). As for adult stem cells, a committee in the Ministry of Health, Labour, and Welfare (MHLW) is now working to establish a set of guidelines for clinical research using human adult stem cells. However, the committee has been struggling with a quite sensitive issue, i.e., use of somatic stem cells derived from dead fetus by miscarriage or by abortion. It will hopefully finalize the guidelines before the end of this year. Having been involved in every step of the above-mentioned stages, I will present an analytical overview of Japanese regulatory framework in this field together with its particularities.

A-12

Legal and Ethical Aspects of Human Embryonic Stem Cell Research in Europe and Israel. SHARON GERECHT-NIR. Harvard-M. I. T. Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139. Email: gerecht@mit.edu

In 1998, researchers reported that embryonic stem cells (ESCs) could be derived from early human embryos and be utilized to develop cell replacement therapies. Clinical use of human ESCs in transplantation medicine is the ethics of using embryos therefore raising a series of ethical and public-policy questions that are now being confronted by international societies and religions. Currently, a countless of international laws and regulation prohibits a systematic, singular point of view, requiring a nation-by-nation analysis. Part of the problem is polarized community opinion on the use of human ESCs for research. This talk highlights the current legislation and regulation of human ESC research in Europe and Israel, which vary in cultural positions and where major scientific activities are taking place. This talk further examines the different approaches and discusses certain concerns that have been raised regarding this novel type of research, mainly due to the lack of a universal approach in relation to the regulation of hESC research. This would allow the understanding of current trends in legal and ethical research study in Europe and Israel, shedding light on the duties and restraints of researchers and their financing agencies.

A-13

Stem Cells and the FDA: Regulatory Considerations for Proceeding to Clinical Trials. DONALD W. FINK, JR. Office of Cellular, Tissue and Gene Therapies, Division of Cell and Gene Therapies, Center for Biologics Evaluation and Research, US-Food and Drug Administration. 1401 Rockville Pike/Suite 200N, Mail Code: HFM-710, Rockville, MD 20852-1448. Email: finkd@cber.fda.gov

Cellular biologic therapies either consisting of or derived from embryonic, fetal or adult stem cells (stem cell-based therapies) may provide effective treatments for current unmet medical needs that necessitate replacement, restoration, repair, or regeneration of damaged or diseased tissues and organ systems. In addition to direct therapeutic efficacy achieved through seeding and repopulating of areas ravaged by trauma and disease, stem cells are also projected to serve as biologic vehicles that may be engineered to deliver functional genes and gene products to discrete target sites where de-generative damage and disease is the consequence of genetic anomalies. Moreover, there is growing recognition that tissue-specific in vitro differentiation of stem cells could result in generation of important new model systems suitable for investigating the underlying biological basis of disease as well as providing critical testing paradigms to screen potential lead candidate pharmaceuticals for toxicological safety and putative therapeutic efficacy. Expansion of ongoing science-driven efforts to identify and critically assess issues pertaining to stem cell safety is vitally important to achieving the ultimate goal of producing novel cellular therapies that are both safe and effective. The challenge of drawing upon all available scientific evidence to assess and gage the safety of biologics produced from stem cells is the responsibility of the Center for Biologics Evaluation and Research (CBER) within the Food and Drug Administration (FDA). The safety and efficacy review of stem cell-based therapies is the responsibility of the Office of Cellular, Tissue, and Gene Therapies (OCTGT), CBER. The explicit mission of CBER is to ensure the safety, purity, identity, potency and efficacy of new biologic therapies through a review process that is founded squarely on scientific principles thus making available to the public innovative new treatments in as timely a manner as is feasible. This presentation will familiarize the listener with regulatory expectations for the development of nascent therapies comprised of stem cells, including those derived from human embryonic stem cells.

A-14

Primary Strategies for Master and Working Cell Line Stock Development. ROBERT J. HAY. Vitro Enterprises, Inc., Bealton, VA 22712. Website: www.vitroenterprises.com

This presentation will review background material to illustrate the critical need for application of stepwise characterizations on all cell lines to be used for research and production purposes. Ideally, one would obtain a representative *Master Stock* aliquot of an existing cell line from one of the many established Biological Resource Centers. Example National Centers include the ATCC, CIMR, or others in the USA; ECACC in the UK; DSMZ in Germany; CBCCTCC in Wuhan, China; NFATCC in Poonna, India; IFO in Osaka, Japan; and the KCLRF in Seoul, Korea. Use of cell lines from these and related initial sources ensures comparability over the years. Contact and procedural online information from these facilities will be made available during the presentation. Additional major important characterizations need to be applied subsequently as outlined elsewhere both in this series of lectures and also in later characterization sections. *Master Seed Stocks* would be prepared and fully characterized for production of authenticated cell line populations. With these populations, confirmation of cellular identities will also be essential. The absence of microbial and viral infections must be assured experimentally. Methodologies for all of these will need to be performed via procedures supported by pertinent CFR documentation. *Master Working Stocks* would be generated and recharacterized as necessary. The general expansion/characterization strategy will be discussed and illustrated through this and related presentations at the conference.

A-15

What Are cGMP and ISO and What Do They Mean for Cell Banking? P. GRAY and L. Jacobsen. Roche Applied Science, Indianapolis, IN 46250. Email: paula.gray@roche.com

Federal and local government agencies provide requirements to medical device, pharmaceutical, and biotechnology companies regarding the development and manufacturing of their products. These come in the form of regulations, guidelines, and points to consider and are necessary for the production of safe and reliable products. While many different regulations exist, the customer's application of the product dictates which are required to be followed. For this reason, many companies choose to be certified to a standard in conjunction with complying with regulations. Standards have been created to outline how a company could organize and document their systems in a fashion that would allow them to produce reliable and safe products. This presentation will identify parts of the cGMP regulations, provide a general overview of the ISO 13485 standard, and discuss where the two overlap. Some specific examples of how this will apply to cell banking will be shared at the end.

A-16

Characterization and Testing of Banked Cell Substrates Used to Produce Biological Products. LEONARD J. SCHIFF. Department of Biopharmaceutical Services, Charles River Laboratories, 10091 Dudley Drive, Ijamsville, MD 21754. Email: lschiff@bps.criver.com

A critical component in controlling the production of biological products derived from human and animal cell lines is the characterization and testing of banked cell substrates. The objective of characterization/testing of banked cell substrates is to confirm the identity, purity and suitability of these cells for manufacturing use. In this presentation, I will discuss the qualification of cell banks including tests for cell identity, endogenous and adventitious microbial contaminants (bacteria, fungi, mycoplasma and viruses). For cells producing recombinant DNA-derived products, analysis of the expression construct at the nucleic acid level (genetic stability) will also be discussed.

A-17

Cell-based Models and Technologies for Space Environment Monitoring. S. R. GONDA^{1,2}, C.T. Culbertson³, E. Behraves⁴, J. L. Huff⁵, R. Pollanen⁶, H. Wu^{5,2}, N. R. Pellis^{6,2}, and J. M. Ramsey⁷. ¹Advanced Biotechnology Laboratory; ²NASA/ JSC, 2101 NASA Parkway, Houston, TX 77058; ³ Department of Chemistry, Kansas State University, Manhattan, KS 66506; ⁴USRA, Department of Space Life Science, 3600 Bay Area Blvd, Houston, TX 77058; ⁵Human Radiation Biodosimetry Laboratory; ⁶Space Life Science Directorate; and ⁷Department of Chemistry Campus, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599. Email: sgonda@ems.jsc.nasa.gov

NASA's exploration of space, the solar system and beyond will expose biological systems to hostile space and planetary environments for prolonged periods. Novel combinations of stressors and selective pressures will affect all life that has originated from this planet. Cells respond to the environment in specific and reproducible ways that are often predictive of functional consequences, and, as bioreporters, can provide an assessment of combinatorial outcomes from numerous simultaneous environmental factors. Multiple events can be probed at the organism, genetic and molecular level, enabling a complete assessment of potential synergistic stressor effects. The concept of microfluidics has significantly influenced the design and the implementation of modern bioanalytical systems due to the fact that these miniaturized devices can integrate multiple chemical processing and handling steps in a much more efficient way than conventional instruments. This results in several advantages including reduced reagent volumes, fully automated biochemical analyses, parallel processing capabilities for system redundancy, and remote operation. These properties greatly enhance the value of microfluidic systems to NASA for use on small spacecraft with limited resources for long duration unmanned environmental monitoring missions. Our approach is to create biosentinels by integrating genetically engineered prokaryotic and eukaryotic cells, microfabricated fluidic bioreactors and analytical technologies, and bioinformatic systems for acute and long-term space monitoring. We will present results of: (i) genotoxic alterations at the gene and molecular level following exposure of 3D tissue-like assemblies containing high density transgene targets to high energy, ionizing space radiation; (ii) whole cell-based bioluminescent prokaryotic bioreporters exposed to combinations of radiation and chemical stressors, and (iii) performance of microanalytical systems in NASA-relevant low-gravity tests aboard the KC-135 aircraft. Supported by NASA 101-31-GN-DA and T-8199W.

A-18

Three-dimensional Tissue Assemblies: Novel Models for the Study of Infectious Disease. C. A. NICKERSON. Department of Microbiology and Immunology, Tulane University Health Sciences Center, New Orleans, LA 70112. Email: cnicker@tulane.edu

Three-dimensional (3-D) cell culture is a powerful tool for investigating the host-pathogen interaction. That is because cells cultured in 3-D model the tissue-like organization and physiology of *in vivo* tissues. We have used innovative bioreactor technology designed at NASA, the rotating wall vessel (RWV), to establish biologically meaningful 3-D cultures of human cells and tissues to study the interaction between host and pathogen. The growth conditions in the RWV provide an optimized form of suspension culture that allows cells to aggregate, grow three-dimensionally, and differentiate into highly specialized tissues. We were first to report the generation, validation, and use of 3-D human tissue aggregates cultured in the RWV as a model for microbial infectivity by a bacterial pathogen. We cultured the human small intestinal epithelial cell line Int-407 in the RWV and showed that the cells formed well-differentiated 3-D tissue assemblies that exhibited apical and basolateral polarity, extensive and well-formed tight-junctions and desmosomes important in the establishment and maintenance of normal epithelial structure, and also produced mucous. These *in vivo*-like phenotypes were not observed in Int-407 cells cultured as monolayers. When used as models to study aspects of Salmonella-induced enteric disease, we observed remarkable differences in adherence, invasion, apoptosis, cytokine profiles, and tissue pathology between the 3-D assemblies and their monolayer counterparts. Many of these differences appear to be more reflective of an *in vivo* infection, which supports our contention that by virtue of their physiological relevance, 3-D cell models hold tremendous potential for infectious disease research. Based on these studies, we have generated a variety of different 3-D cell culture models of other human tissues that recapitulate many aspects of cellular structure, differentiation, and function that occur *in vivo*, and which are currently being used by us in infection studies. These models include colon, lung, placenta, bladder, and periodontal ligament. These 3-D cultures are being used to model infections caused by Salmonella and Vibrio sp (colon); Pseudomonas aeruginosa and Klebsiella pneumoniae (lung); human cytomegalovirus (placenta), uropathogenic Escherichia coli (bladder), and Porphyromonas gingivalis (ligament).

A-19

Rotating Bioreactor Studies of Cardiac and Skeletal Tissue Constructs. L. E. FREED and G. Vunjak-Novakovic. Harvard-MIT Division of Health Sciences and Technology, MIT, Cambridge, MA 02139. Email: lfreed@mit.edu

Rotating bioreactors, which simulate some aspects of microgravity, were used to study cardiac and skeletal tissue constructs comprised of cells and biomaterial scaffolds. In one study, we hypothesized that cardiac cells cultured in tissue constructs would exhibit better molecular and electrophysiological properties than the same cells cultured in confluent monolayers. Thin slices of native heart tissue were also assessed to provide a baseline. Construct levels of contractile and gap junctional proteins were 50% as high as native heart, whereas monolayer levels of the same proteins were only 10-20% as high. Construct action potential duration (APD) was 1.8-fold higher than native heart, whereas monolayer APD was 2.4-fold higher. Pharmacological studies provided a possible explanation for the electrophysiological differences. In another study, we hypothesized that integrative repair of cartilage constructs depended on the type of cell, scaffold, and adjacent tissue (cartilage or bone). Significant effects of cell type on construct adhesion ($p < 0.001$) and of scaffold type on construct adhesion ($p < 0.001$), modulus ($p = 0.014$), and amount of glycosaminoglycan (GAG) ($p < 0.001$) were observed, with the best results obtained if scaffold degradation time matched the rate of chondrogenesis. Likewise, type of adjacent tissue significantly affected construct adhesion ($p < 0.001$), modulus ($p < 0.001$), and GAG ($p < 0.001$), with better results obtained for constructs cultured adjacent to bone than cartilage. In the present studies, rotating bioreactors provided a high fidelity *in vitro* model for studies of cardiac and skeletal tissue constructs. The same platform could potentially be used to assess the efficacy of new drugs to prevent muscle and bone loss in astronauts during spaceflight.

A-20

Gene Expression Alterations in Activated Human T-cells Induced by Modeled Microgravity. N. E. WARD¹, N. R. Pellis², S. Risin³, and D. Risin². ¹Wyle Life Sciences, Houston, TX; ²NASA-Johnson Space Center, Houston, TX; and ³University of Texas-Houston Medical School. Email: nward@ems.jsc.nasa.gov

It is recognized that in the microgravity of spaceflight there is a decline in cellular immune function. One of the methods employed to investigate microgravity effects in ground based systems is the NASA Rotating Wall Vessel (RWV) bioreactor, in which cells are exposed to a continuous free fall situation under low shear stress and randomized gravitational vector resulting in a simulated microgravity model. To study the microgravity adaptation of human immune cells, we have utilized the RWV bioreactor and microarray analysis to identify associated gene expression changes. In this work, chronically CD3 and IL2 activated T-cells were cultured in 1g (static) and simulated microgravity (RWV) conditions for 24 hours. Microarray analysis was performed utilizing Affymetrix Gene Chips. To decrease biological variation and aid in detecting microgravity-associated changes, microarray analysis experiments were performed in triplicate utilizing T-cells obtained from different blood donors. Exposure to simulated microgravity resulted in the alteration of 89 genes, 10 of which were up-regulated and 79 down-regulated. In separate experiments activated T-cells were cultured in 1g and simulated microgravity conditions and then stimulated for three hours with phytohemagglutinin (PHA). Under these conditions, microarray analysis revealed gene expression changes in 85 genes, 20 of which were up-regulated and 65 down-regulated. The altered genes were categorized by the associated pathways and viewed graphically through histogram analysis. Separate histograms of each pathway were then constructed identifying individual gene expression fold changes. This provided base information for evaluating the possible role of genes demonstrating altered expression in lymphocyte response to microgravity at the molecular level. (Supported by NRA OLMSA-02 and NSCORT NAG5-4072 grants).

A-21

Approaches for Developing Novel Probes/Loci for PCR-based Detection of Pathogens. VIPIN K. RASTOGI¹ and Tu-chen Cheng². ¹MAP Team, R & T Directorate, US Army - Edgewood Chemical & Biological Center, APG, MD 21010 and ²GEO-Centers, Inc. Gunpowder Club, APG, MD 21010. Email: vipin.rastogi@us.army.mil

Bioterrorism and the growing challenge of natural emerging infectious diseases are flip sides of the same coin. Both phenomena manifest themselves in the form of an unusual outbreak of infectious disease - one that must be rapidly detected and decontaminated to minimize the harmful consequences. DNA-based detection technology of Bio-Warfare Agents, such as *Bacillus anthracis*, the causative agent of anthrax, is among the most sensitive (around 10-100 spores detected) approach known to-date. Current field detection for *B. anthracis* relies principally on amplification of virulence determining genes. Lack of a positive control in the assay protocols for field detection of BWA precludes validation of the test results. A logical approach to address this problem is to include additional DNA markers, preferably chromosomal located in the detection protocol. Amplification of both virulence genes and unique chromosomal in the field-testing of suspected samples is expected to validate the field test results. To this end, we have identified five unique chromosomal regions of *B. anthracis* by random primer amplification. *Burkholderia pseudomallei* and *B. mallei*, respectively are the causative agents of melioidosis and glanders, primarily in animals (both pathogens), and in humans (commonly the former but sporadically the latter as well). The two pathogens are gram-negative, facultative anaerobe, motile bacilli. There is no known vaccine, and the treatment with antimicrobials is protracted because of natural resistance of these pathogens to commonly used antibiotics. There is currently no real-time PCR assay for detection of both of these pathogens. Primers and probes corresponding to specific genomic regions were designed and tested in an attempt to develop RT-PCR assays for detection of both these pathogens. Here, we report results on: 1) the identification of DNA sequences of anthrax chromosome regions. Primer sets have been designed and results based on their use in detecting anthrax in environmental samples will be summarized; and 2) successful development of highly specific RT-PCR assays for detection of both *B. mallei* and *B. pseudomallei* DNA.

A-22

Invertebrate-specific Assessment of the Effect of Endocrine Disrupting Chemicals Using the Estuarine Mysid *Neomysis Integer*. A. GHEKIERE¹, N. Fockede², T. Verslycke^{1,3}, M. Fenske⁴, T. Soin⁵, G. Smaghe⁶, and C. R. Janssen¹. ¹Ghent University, Laboratory of Environmental Toxicology and Aquatic Ecology, J. Plateaustraat 22, B-9000 Ghent, BELGIUM; ²Ghent University, Marine Biology Section, Krijgslaan 281/S8, B-9000 Ghent, BELGIUM; ³Current address: Biology Department MS#32, Woods Hole Oceanographic Institution, Woods Hole, MA 02543; ⁴University of Exeter, Hatherly Laboratories, Exeter, EX4 4PS, Devon, UK; ⁵Current address: Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK; and ⁶Ghent University, Laboratory of Agrozoology, Coupure links 653, B-9000 Ghent, BELGIUM. Email: an.ghekiere@ugent.be

The occurrence of endocrine disrupters (EDs) in the environment and the potential effects on wildlife species is receiving increased public attention. Although invertebrates account for roughly 95% of all animals, surprisingly little research has been performed to understand the effects of EDs on these organisms and to evaluate their value in signalling the occurrence of environmental disruption through EDs. Increased knowledge of invertebrate-specific endocrine regulated processes and their disruption by chemicals is, however, required. Disruption of ecdysteroids, the molting hormones, for instance, which play a major role in reproduction and embryogenesis, would have profound effects on many aspects of organismal functioning. In this context we studied the effects of EDs on the molting cycle and *in vitro* embryonic development of the estuarine mysid *Neomysis integer* (Crustacea, Mysidacea). Additionally, a new ELISA was developed to quantify vitellin (egg yolk protein), which enabled us to evaluate effects on vitellogenesis. This research will be illustrated by discussing the effects of the insecticide methoprene, a juvenile hormone analogue, on *N. integer*. Furthermore, the use of insect cell lines to screen EDs with (anti-)ecdysteroidal activity will be discussed.

A-23

Novel In Vitro Cell Culture Model of Adult Mammalian Spinal Cord Cells. MAINAK DAS¹, Neelima Bhargava¹, Swanand Patil², Lisa Riedel¹, Peter Molnar¹, Sudipta Seal², and James J Hickman¹. ¹Nanoscience Technology Center and ²Advanced Materials Processing and Analysis Center, University of Central Florida, Orlando, FL 32826. <http://www.nanoscience.ucf.edu/contact.html>. Email: mdas@mail.ucf.edu

This work addresses one of the most intriguing questions of modern neuronal medicine: How we could protect adult mammalian spinal cord nerve cells, either following injury or in neurodegenerative diseases. One of the key prerequisite in order to address this question is to create a serum-free defined *in vitro* cell culture model of adult mammalian spinal cord cells. Such a model will not only help in understanding the adult spinal cord neuron survival but will help in screening different novel drugs for spinal cord repair and neurodegeneration. Recently we have developed a serum-free *in vitro* cell culture model that promotes the growth, regeneration and long-term survival of dissociated adult mammalian spinal cord neurons and glial cells. The system comprises of a synthetic, cell growth promoting silane substrate, N-1 [3-(trimethoxysilyl) propyl] diethylenetriamine (DETA) coated on glass and a chemically defined novel serum-free medium, supplemented with specific growth factors. This presentation shall document our methodology of developing such a culture system, and discuss the progress we have made in the following areas using this model: 1. **Mammalian spinal cord injury**. 2. **Degenerative diseases of spinal cord**. 3. **Remyelination**: The factors involved in remyelination of injured neurons of spinal cord. 4. **Neurogenesis**: The process of neurogenesis in the adult spinal cord and its implications in spinal cord injury and neuro-degenerative diseases. 5. **Nanomedicine**: Use of engineered anti-oxidant nanoparticles (Cerium Oxide nanoparticles) in spinal cord regeneration. (Collaboration with Prof. Sudipta Seal² <http://people.cecs.ucf.edu/sseal/>).

A-24

Mechanisms Regulating Adult Hippocampal Neural Stem Cells. H. J. SONG. Institute for Cell Engineering, Departments of Neurology and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205. Email: shongju1@jhmi.edu

Neurogenesis, the birth of new neurons from progenitors, occurs throughout life in the dentate gyrus of the hippocampus in all mammals examined, including humans. Understanding mechanisms regulating adult neurogenesis will not only advance our general understanding of neural development, but may also leads to stem cell based strategies for repair of the adult central nervous system (CNS) after injury or degenerative neurological diseases. We have isolated multipotent neural progenitors from the hippocampus of adult rats, mice and humans. These neural progenitors can be expanded and maintain their multipotentiality in defined medium without serum with addition of specific growth factors. Using immunocytochemistry, electron microscopy, FM-imaging and electrophysiology, we have shown that adult neural stem cells retain the ability to give rise to electrical active and synaptically integrated neurons with all essential characteristics of mature CNS neurons. Using this culture system, we are investigating the cellular and mechanisms regulating sequential steps during adult neurogenesis, from proliferation, fate specification and neuronal subtype differentiation of adult neural progenitors, to neuronal maturation and synaptic integration by newborn neurons. Results from the latest studies will be discussed.

A-25

Rare Event Detection & Multiplexing in Neural Stem Cell Biodiscovery. S. E. BOUCHER. Invitrogen Co., GIBCO Cell Culture R&D, Grand Island, NY 14072. Email: shayne.boucher@invitrogen.com

The need for detection and analysis of multiple and rare analytes is critical in neural stem cell research. These include transcription factors, signaling molecules and phenotypic changes. Often, novel and integrative strategies are required for unraveling the complex interplay of these genes, mRNAs and proteins. This talk will highlight i) serum-free culture systems for signaling studies, ii) expression reporter systems for understanding cellular function, iii) RNAi vectors for knockdown of gene expression, iv) lentiviral expression and transfection reagents for production of specific proteins, v) multicolor FISH for detection of mRNAs in cells and tissue, vi) technology for labeling and detection of antibodies from the same species, and vii) enzymatic and superbright fluor reagents for rare event detection. The intent will be to map out a biodiscovery strategy for elucidating gene and cellular behavior in neural stem cells.

A-26

Mechanoregulation of Stem Cell Function. C. S. CHEN. University of Pennsylvania, Department of Bioengineering, 125 31st Street, Philadelphia, PA 19104. Email: chrischen@seas.upenn.edu

The binding interactions between cell surface receptors and local bioactive ligands serves as the principal mechanism by which cells survey their microenvironment and accordingly modulate their behaviors, such as proliferation, differentiation, migration, and suicide. Using microfabrication approaches to engineer cellular microenvironments, we are examining how different cues can be used to regulate cellular functions. We are investigating the interactions between signals from the extracellular matrix, growth factors, intercellular adhesions, and mechanical forces in both endothelial cells and mesenchymal stem cells to understand how environmental signals integrate to regulate cell proliferation and differentiation. Our studies demonstrate that the modulation of proliferation and differentiation by adhesive and soluble cues depend on changes in the actin cytoskeletal tension, and signaling by the Rho family of GTPases. We will discuss our approaches to control compositional chemistry, mechanical properties, architecture, and geometry of surfaces, and how these factors regulate cells. We hope to use these results to improve the interconnect between artificial surfaces with living cells.

A-27

Computer-aided Tissue Engineering: A Modern Engineering Approach for Scaffold Design and Fabrication. WEI SUN. Laboratory for Computer-Aided Tissue Engineering, Department of Mechanical Engineering and Mechanics, Drexel University, 3141 Chestnut Street, Philadelphia, PA 19104. Email: sunwei@drexel.edu, <http://www.mem.drexel.edu/cate>

The revolution in biological sciences and bioengineering has created an environment in which the advances in the life sciences are not only amenable to, but require, the active participation of engineering design and manufacturing. This revolution, along with the advancements of modern design and manufacturing, biomaterials, biology and biomedicine have emerged a new field of Computer-Aided Tissue Engineering (CATE). CATE enables the application of advanced computer-aided technologies and biomechanical engineering principles to derive systematic solutions for complex tissue engineering problems. This presentation will introduce the recent development in computer-aided tissue engineering and its application to design and fabrication of tissue scaffolds. The presentation will include three topics: 1) computer-aided tissue modeling, including representation and modeling of 3D tissue and anatomic system, bio-modeling, 3D reconstruction and application to surgical planning and simulation; 2) computer-aided tissue scaffold informatics and biomimetic design, including classification and characterization of tissue structural and morphological properties, multi-scale modeling, design and fabrication of biological tissue systems and scaffolds at different hierarchical levels, and biomimetic design of load bearing bone and cartilage tissue scaffolds and replacements; and 3) computer-aided bio-manufacturing manufacturing, including the development of proprietary multi-nozzle biopolymer deposition system for freeform fabrication of tissue scaffolds and cell seeded tissue precursors.

A-28

Nanotopographic Tools for Vascular Tissue Engineering. JEFFREY T. BORENSTEIN¹, Christopher J. Bettinger^{1,2}, David J. Carter¹, Brian K. Orrick¹, and Robert Langer². ¹Draper Laboratory, Cambridge, MA 02139 and ²Massachusetts Institute of Technology, Cambridge, MA 02139. Email: jborenstein@draper.com

The role of mechanical forces in governing cell signaling and cell-substrate interactions is gaining increasing recognition, with applications ranging from fundamental studies of the origin of disease to drug discovery and regenerative medicine. In particular, mechanical forces such as shear stress and surface topography can be used to probe endothelial cell behavior. Substrate topography effects have been characterized for numerous cell types, using a variety of micron-scale features machined into substrates such as titanium, ceramics, silicon, and silicone rubber. The utility of these studies has been limited by the fact that while imprinting of micron-scale features on substrates is straightforward, investigations of the basement membrane which underlies epithelial and endothelial reveals that the signature topography is closer to 100 nanometers. In addition, virtually all of these experiments have been conducted on rigid, permanent substrates suitable for laboratory studies, whereas the use of nanoscale mechanotransduction as a tool for engineered tissues will likely require the use of biodegradable substrates. Here we present the results of an investigation of endothelial cell interactions with nanoscale features imprinted into a biodegradable substrate, PolyGlycerol Sebacate (PGS). Features ranging from 400 - 900 nanometers are shown to have powerful effects on the orientation and aspect ratio of bovine aortic endothelial cells cultured on PGS substrates. These results suggest that nanotopographic substrate modification can be used as a tool for in vitro generation of vascular tissues.

A-29

Tissue Models as Replacements for Animal Experimentation. S DHURU-VAKUMAR. People for the Ethical Treatment of Animals (PETA), Norfolk, VA 23510. Email: SadhanaD@peta.org

Although the field of tissue engineering had its provenance in growing tissues for transplantation, there is a substantial and escalating interest in using tissue constructs as *in vitro* research systems. Traditionally, basic and drug-related research had a strong focus on studying animals as surrogates for humans even though attempts to replicate human diseases in animals often fail. Scientists are increasingly turning instead to human-based *in vitro* models because they are generally less variable, more reproducible, less expensive, quicker, more scalable, do not suffer from error due to species differences, and last but not least, they satisfy the mounting public pressure to reduce animal use for ethical reasons. Amongst the most promising of the non-animal alternative technologies are human-based tissue models. These models represent the best possible chance to mirror *in vivo* human biology if developed appropriately, although achieving physiological relevance remains a challenge and depends on advancing our detailed understanding of human biology. Nonetheless, this field is burgeoning with models in development for many types of organs and tissues and refinements are continually improving physiological relevance and predictive power.

A-30

Physical Effects on Chondrocytes: Basic Science to Tissue Engineering. CLARK T. HUNG. Department of Biomedical Engineering, Columbia University, New York, NY 10027. Email: cth6@columbia.edu

The prevalence of osteoarthritis articular cartilage, coupled with the poor intrinsic healing capacity of this tissue, has given rise to a great demand for an articular cartilage substitute. As the bearing material of diarthrodial joints, articular cartilage has remarkable functional properties that have been difficult to reproduce in tissue engineered constructs. The underlying premise for our work is that the application of physiologic loading will promote development of cartilage tissue engineered constructs having functional load-bearing tissue properties. In this context, I will describe two complementary research thrusts in my laboratory, one aimed at understanding chondrocyte mechanotransduction and one aimed at efforts toward the application of deformational loading for cultivating functional tissue constructs. Study 1 will describe a system that our research team has developed to study for the first time the cyclic deformational loading response of cultured chondrocytes using osmotic loading and microfluidic technology. Osmotic loading also offers an opportunity to deform the chondrocyte without physical "touching" of the cell with a probe or matrix deformation. Calcium levels ($[Ca^{2+}]_i$) can be monitored cell-by-cell using fura-2 fluorescence microscopy, providing a rapid and dynamic measure of cell signaling as well as cell size change in response to applied loading. Study 2 will focus on efforts exploring the novel preconditioning of bovine chondrocyte-seeded agarose hydrogel constructs using deformational loading, arguably one of the most prominent components of the chondrocyte physical environment *in vivo*. The beneficial effects of applied physiologic loading are mediated by construct cell seeding density and media supplementation with growth factors.

A-31

Stem Cell Behavior in Hydrogels. J. ELISSEFF. Department of Biomedical Engineering, Johns Hopkins University, Baltimore, MD, 21218. Email: jhe@bme.jhu.edu

Adult and embryonic stem cells have received significant attention for their potential in therapeutic applications including tissue engineering. While stem cells have been studied in standard monolayer tissue culture conditions, three dimensional culture in biomaterials is often required for tissue development and therapeutic repair applications. Hydrogels are three dimensional networks that can encapsulate cells. We have encapsulated adult and embryonic stem cells in hydrogels for applications in skeletal tissue engineering. Embryoid bodies created from embryonic stem cells were encapsulated in hydrogels and incubated *in vitro* in chondrogenic conditions. Tissue-specific transcription factors and matrix molecule expression increased and cartilage-like tissue was formed only when cells were cultured in hydrogels compared to monolayer culture. Furthermore, stem cell response to growth factors in the 3D hydrogels significantly differed from stem cells cultured in standard monolayer culture. Stem cells encapsulated in hydrogels also influence the behavior of differentiated cells in a bilayered coculture system. Stem cells in hydrogels cultured adjacent to differentiated chondrocytes in bilayered hydrogels produced a stimulus to promote chondrocyte proliferation and differentiation. In sum, biomaterials will play an important role in stem cell research and development.

A-32

Donor Age, Cell Density, and Complimentary Roles of Cytoskeletal Elements in the Contraction of Fibroblast-populated Collagen Gels. R. A. REDDEN and E. J. Doolin. Department of General Pediatric Surgery, Children's Hospital of Philadelphia, Philadelphia, PA 19104. Email: redden@email.chop.edu

Introduction. The healing of deep skin injuries is often plagued by excessive scarring and contracture, and patient age has a significant influence. Collagen content has been most thoroughly examined, but a crucial, poorly understood factor is dynamic, inter- and intracellular force generated by fibroblasts. This research used an *in vitro* model, the contraction of fibroblast-populated collagen gels (FPCG), to investigate contractility of fibroblasts, specifically the contribution of cytoskeletal elements and the influence of age and cell density. **Methods.** FPCGs were created and seeded with normal human dermal fibroblasts. The gels were polymerized and floated in growth media. Digital images were taken daily and gel area was calculated. **Research Design.** Two variables were investigated: 1) cell density (50k, low; 500k/ml, high), 2) patient age (neonatal, adult, and senior donors). The contribution of cytoskeletal elements to FPCG contraction was determined using microtubule and microfilament disruptors (colchicine and cytochalasin, respectively). **Results/Conclusions.** FPCG contraction is complicated and bimodal. In all cases, FPCGs seeded at high cell density (HD) contracted earlier and more rapidly than those at low cell density (LD). LD-FPCGs showed a delay of several days before contraction began. FPCGs seeded with neonatal fibroblasts contracted faster than those seeded with either senior or adult fibroblasts. Finally, cytoskeletal disruption revealed differential, complimentary contributions of microtubules and microfilaments. Both drugs were inhibitory, but affected different temporal aspects of contraction, most notably in HD-FPCGs. We hypothesize that HD-FPCGs contract through different primary mechanisms than LD-FPCGs, and that age affects fibroblast contractility.

A-33

A Neuronal Network-based Biosensor for Detection of Environmental Threats. THOMAS J. O'SHAUGHNESSY. Center for Bio/Molecular Science and Engineering, United States Naval Research Laboratory, Washington, DC 20375. Email: tjo@cbmse.nrl.navy.mil

Contamination of the environment by toxic compounds is a major problem for many military and civilian agencies. The range of compounds of concern includes heavy metals, organophosphates and other pesticides. This broad spectrum of compounds, with the possibilities of adverse combinations as well as unknown toxicants, makes specific testing difficult and requires a generic type of detector. To address these issues, a portable cell-based biosensor, utilizing networks of mammalian neurons grown on microelectrode arrays as the sensor element, has been developed at the Naval Research Laboratory (NRL), in collaboration with the University of North Texas. The device itself consists of temperature control and fluidics subsystems necessary to maintain the neuronal networks in a shipping/recording cartridge, while simultaneously recording the electrical activity of the neurons. The system detects the action potentials from multiple electrodes of the microelectrode array simultaneously through non-invasive, extracellular recording techniques. Analysis of changes in frequency and patterns of these signals can then be used to determine if the environmental sample, typically potable water, contains neuroactive or neurotoxic compounds that would warrant further investigation.

A-34

APC-biosensor: The Use of Dendritic Cells and Monocytes for Early Detection of Exposure to Toxic Substances. KEITH D. CRAWFORD, Harvard University, Brigham and Womens Hospital, Orthopaedics/Molecular Orthopaedics, BLI-152, 221 Longwood Ave., Boston, MA 02115. Email: Crawford@rics.bwh.harvard.edu

Infectious agents have been and are likely to be used again for purposes of bio-terrorism. They may be used either as wild type strains or genetically modified strains, which are manipulated to enhance their harmful potential. The body's first line of defense against such agents is the immune system, which innately or adaptively protects against pathogens. A subset of immune cells, dendritic cells (DC) and monocytes (Mo), are APCs (Steinman 1991). These specialized immune cells are in all tissues of the body, especially at mucosal barriers. Once exposed to infectious or noninfectious agents, they phagocytize and transport pathogens to primary immune tissue. Once localized at this site, pathogen are degraded into peptides and presented to T cells. These T cells are responsible for mediating adaptive immunity, which involves antigen-specific cytotoxic T cell expansion and immunoglobulin production by B cells. In some case, pathogens evade this degradation and use the APC as site of replication, as described for Marburg virus (Lambrecht 2001, Bosio 2004). By sequestering antigens, APCs act as bio-concentrators rendering them ideal cell populations to detect micro-organism-specific protein and gene expression profiles. Previous studies have demonstrated, that DCs respond to pathogen exposure by altering gene expression. This altered gene expression is unique to the pathogen to which the DCs are exposed (Huang 2001). Thus, these unique changes in gene expression can be used to discriminate different pathogens (Reis & Sousa, 2001). We have taken this concept one step further and have successfully demonstrated the existence of pathogen-specific protein profiles with Surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS). SELDI-TOF-MS is a recent technology that allows high-throughput proteomic analysis with limited material requirements (Barzaghi, 2004). Our preliminary data demonstrates that DC and Mo co-cultured with bacteria, endotoxin, viruses, or genetically modified viruses have distinctive protein/peptide profiles, which could be used to for early detection and discrimination for pathogen exposure. Future research should provide the foundation for the development of a means of accurate, rapid, and early detection of exposure to infectious pathogens using gene array and/or MS-protein profiling technologies coupled with APC-biosensor concept. In addition, identification of pathogen-regulated proteins may improve our understanding of their role in pathogenesis as the identification of possible targets for immunotherapy.

P-1

Engineering of N and S Metabolism. CHAMPA SENGUPTA-GOPALAN, Suman Bagga, Carol Potenza, and Jose Ortega. Department of Agronomy & Horticulture, New Mexico State University, Las Cruces, NM 88003. Email: csgopala@nmsu.edu

Alfalfa is the most important forage legume in the United States, providing quality protein for animal feed. Our research is focused on two major aspects dealing with the improvement of alfalfa: nitrogen use efficiency and increasing methionine (Met) level. Nitrogen is the inorganic nutrient that plants require in greatest quantity and that most frequently limits productivity in agricultural systems. Glutamine synthetase (GS), the first key enzyme in ammonia assimilation, could represent a key component of nitrogen use efficiency and yield. Thus one of our objectives has been to manipulate GS in a tissue-specific manner in transgenic alfalfa. However, GS is very highly regulated, and thus a major focus of our research is to understand the regulatory mechanisms underlying its expression. We will present data showing the multistep regulation of GS and the effect of modulating GS activity on plant performance. We will also present data that links nitrogen assimilation with carbon metabolism and shed some light on global changes in gene expression associated with increased GS activity. In our effort to increase Met level in alfalfa, we have used a powerful combination of both "source" and "sink" derived transgenes to understand the flow of metabolites through this pathway, and the ultimate destinations of Met and its derivatives within the plant. We have used the corn genes for Met-rich delta- and beta-zeins to provide a stable Met sink and are targeting multiple steps in the Met biosynthetic pathway to increase free Met pool. Our approach includes upregulating the gene for cystathionine gamma-synthase and/or downregulating threonine synthase and SAM synthase. We will present some data on metabolite analysis of these transformants and the feasibility of our approach to increase Met level in alfalfa.

P-2

Terpenoid Engineering from Plants to Bacteria. S. T. WITHERS and J. D. Keasling. Department of Chemical Engineering, University of California-Berkeley, Berkeley, CA 94720. Email: keasling@berkeley.edu

As the largest class of natural compounds, terpenoids are an attractive target for metabolic engineering in *E. coli*. The terpenoid artemisinin is of particular interest for its exceptional anti-malarial activity. Current methods extract artemisinin from *Artemisia annua* in a process that results in a drug much too expensive for the developing world. In an effort to improve access to this drug we are engineering a strain of *E. coli* to produce artemisinin at a reduced cost. We have developed strains capable of producing an array of plant terpenoid products including mono-, sesqui-, and diterpenes, although at low levels due to poor terpene synthase expression. Production of the sesquiterpene precursor to artemisinin, amorpha-4,11-diene, was accomplished by constructing a synthetic amorphaadiene synthase (ADS) gene. To test if the yield of amorphaadiene was limited by the availability of the substrate farnesyl diphosphate (FPP) we expressed the mevalonate pathway genes from *Saccharomyces cerevisiae* along with the IPP/DMAPP isomerase and FPP synthase from *E. coli* from a plasmid. The expression of the mevalonate pathway genes dramatically increased the FPP levels. The engineered strain was found to produce terpene precursors at such a level sufficient to inhibit growth in the absence of the amorphaadiene synthase. Co-expression of the mevalonate genes with ADS restored normal growth. The combined efforts of metabolic engineering and culture optimization have already resulted in a 10⁶-fold improvement in terpenoid production. Work continues to increase the flux through the mevalonate pathway upstream of mevalonate and to identify the remaining genes in the artemisinin biosynthetic pathway.

P-3

Metabolic Modeling for Metabolic Engineering. D. RHODES. Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907. Email: drhodes@purdue.edu

Metabolic engineering in microbial systems has benefited greatly from the use of a number of mathematical and computational methods for quantifying the flow of biochemical material via metabolic pathways *in vivo*. These methods are routinely used in microbial systems to assess the effects of targeted genetic change on the *in vivo* rates of synthesis of metabolites, and hence to gauge the success or failure of specific genetic interventions aimed at diverting metabolic flux to desired end-products. For the most part these methods rely on assumptions of metabolic (and isotopic) steady-state, and absence of compartmentation of metabolic pools. These methods are difficult to apply to higher plant metabolic systems because metabolic and isotopic steady-state can only rarely be achieved experimentally in higher plants due to large, slowly-turning-over storage pools of polymers and intermediates. Isotope flux analysis models more suited for higher plant tissues involve consideration of the transient flow of isotopic labeled compounds via metabolic networks. The utility of such transient labeling metabolic flux models will be illustrated with examples from isotopic labeling of glycine betaine in transgenic tobacco plants, flux analysis via the benzenoid network in petunia flowers, and flux analysis via amino acid metabolism networks using carbon-13 labeled amino acid precursors. It is anticipated that these models will be of general use in the emerging fields of plant metabolic engineering and functional genomics, and the established field of environmental plant stress physiology.

P-4

Plant Transcription Factors: Gene Families, Family Evolution, Functions with Ectopic Expression, and Applications. N. GUTTERSON. Mendel Biotechnology Inc., 21375 Cabot Boulevard, Hayward, CA 94545. Email: ngutterson@mendelbio.com

Mendel Biotechnology has conducted a large-scale, high throughput program to identify the function of plant transcription factors, using the *Arabidopsis thaliana* complement of transcription factors as a model system. The different families of transcription factors will be surveyed, and the relative expansion of these families reviewed in sequenced genomes. Many families are conserved throughout eukaryotes, although some have been uniquely expanded in plants; several families are unique to plants. General principles of transcription factor function will be illustrated, using well characterized examples, including transcription factors that control floral induction, cold tolerance, and developmental patterning. Lessons from Mendel Biotechnology's screen of over 1700 transcription factors through ectopic expression will be summarized. In particular, the utility of our open-ended, candidate-gene-class approach to identify transcription factors that regulate important agronomic traits such as abiotic stress tolerance and biomass will be illustrated. Finally, a member of the AT-hook transcription factor family that regulates plant growth rate and development will be described by way of example. A deep analysis of the regulatory networks upon which this and other transcription factors act is now being performed in support of crop product development. Future directions for research involving transcription factor function and analysis of genetic regulatory networks will be considered.

P-5

Gene Regulons and Regulatory Networks Involved in Plant Cold Acclimation. MICHAEL F. THOMASHOW. MSU-DOE Plant Research Lab, Michigan State University, East Lansing, MI 48824-1312. Email: thomash6@msu.edu

Plants vary greatly in their responses to cold temperatures. At one extreme are plants from tropical and subtropical regions such as soybean and rice, which suffer injury when exposed to chilling temperatures between 0 and 12° C. In sharp contrast, plants from temperate regions are not only chilling tolerant, but many, such as Arabidopsis and wheat, can survive freezing after exposure to low nonfreezing temperatures, a phenomenon known as “cold acclimation.” Our long range objective is to understand the “genomic basis” of cold acclimation. Our current efforts focus on genes that are induced during cold acclimation. Recent studies with Arabidopsis have established that cold acclimation involves action of the CBF cold response pathway, a regulon of genes controlled by expression of the CBF transcriptional activators. Our current aim is to construct a low temperature “wiring diagram” of Arabidopsis that includes the identification of gene regulons and regulatory networks that have important roles in cold tolerance. In addition, in a collaborative project funded by the NSF Plant Genome Initiative, we are determining whether the Arabidopsis CBF cold-response pathway is highly conserved in plants and whether differences in plant cold tolerance can be traced to differences in CBF cold-response pathways.

P-6

Increased Cuticular Wax Accumulation and Enhanced Drought Tolerance in Transgenic Alfalfa by Overexpression of a Transcription Factor Gene. Ji-Yi Zhang¹, Corey D. Broeckling², Alison B. Blancaflor², Mary Sledge¹, Lloyd W. Sumner², and ZENG-YU WANG¹. ¹Forage Improvement Division, ²Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, OK 73401. Email: zywang@noble.org

Plant cuticular waxes play an important role in protecting aerial organs from damage caused by multiple environmental stresses such as drought, cold, UV radiation, pathogen infection, and insect attack. The identification of leaf wax genes involved in stress tolerance is expected to have great potential for crop improvement. Here we report the characterization of a novel AP2 domain-containing transcription factor gene from the model legume plant *Medicago truncatula*. The gene, designated *WXPI*, is able to activate wax production and confer drought tolerance in alfalfa (*Medicago sativa*), the most important forage legume species in the world and a close relative of *M. truncatula*. The predicted protein of *WXPI* has 371 aa; it is one of the longest peptides of all the single AP2 domain proteins in *M. truncatula*. *WXPI* is distinctly different from the most studied genes in the AP2/ERF transcription factor family, such as *AP2s*, *CBFs*, *DREBs*, *WIN1* and *GL15*. Transcript level of *WXPI* is inducible by cold, ABA and drought treatment in shoot tissues. Overexpression of *WXPI* under the control of CaMV35S promoter led to a significant increase in cuticular wax loading on leaves of transgenic alfalfa. *WXPI* overexpression induced a number of wax-related genes. Transgenic leaves showed reduced water loss and chlorophyll leaching. Transgenic alfalfa plants with increased cuticular waxes showed enhanced drought tolerance demonstrated by delayed wilting after watering was ceased and quicker and better recovery when the dehydrated plants were re-watered.

P-7

Developing a Commercial Somatic Embryogenesis Platform for Large Scale Production of Conifer Seedlings. S. M. ATTREE. CellFor Inc., PO Box 133, Victoria, British Columbia, CANADA, V8M 1R3. Email: sattree@cellfor.com

CellFor Inc., is a technology company serving the Forest Industry and private land owners. The majority of CellFor's employees are located on Vancouver Island, at our research and production facility. CellFor is the first company to commercialize the conifer somatic embryogenesis (SE) production process. CellFor produced about 2 million bare root somatic seedlings in 2004, and production volumes are expected to double or triple each year for the next few years. This creates significant challenges for CellFor's scientists and staff to identify the problems and bottlenecks associated with producing such volumes. At the same time, costs of production are required to decrease significantly. While some costs can be simply reduced by “economies of scale”, there are nonetheless significant challenges to be overcome to reduce direct costs. Plant tissue culture has traditionally been a labor intensive process. Published methods for producing conifer seedlings describe inefficient methods for bulking up tissue, transferring embryos and germinating seedlings *in vitro*, prior to transferring them to the nursery environment. To be efficient it is essential to reduce the labor component of the production process using automation. However, improvements to the overall efficiencies throughout the process, and reducing or eliminating various sources of variability, are also key components. This paper provides an overview of CellFor and the challenges faced, and provides and insight of some of the processes developed to meet these challenges.

P-8

Plum Pox Virus Resistant Plums - A Case Study of the Potentials and Pitfalls of Genetically Engineered Fruit Trees. R. SCORZA and M. Ravelonandro. ¹USDA-ARS Appalachian Fruit Research Station, 2217 Wiltshire Road, Kearneysville, WV 25430 and ²Institut De Biologie Vegetale Et Moleculaire INRA, BP 813383 Villenave d Ornon, FRANCE. Email: scorza@afrs.ars.usda.gov

Rapid progress in fruit tree breeding is limited by long generation cycles (3-20 years), and the high costs associated with large land areas and orchard operations typical of fruit tree breeding programs. High levels of heterozygosity, severe inbreeding depression, complex intraspecific incompatibility relationships, and nucellar embryony add to the difficulties in the breeding of particular fruit species. Combining and selecting for multigenic traits such as high fruit quality and disease and/or insect resistance add to the expense and complexity of fruit tree breeding programs and can further slow the pace of genetic improvement. The culmination of fruit tree breeding programs is the release of improved cultivars. This work can span the lifetime of one to several generations of breeders. Genetic engineering has the potential to revolutionize fruit tree breeding by providing an approach that can specifically target genetic improvements and that offers the possibility of developing novel useful traits. While the technology does not provide a panacea for all of the problems associated with fruit tree breeding, it offers a useful approach to increase the efficiency and effectiveness of breeding programs. In spite of the potential utility of genetic engineering for fruit tree improvement, the technology has not, to date, been widely exploited in these species. Of over 11,000 field tests of transgenic plants in the United States between 1987 and 2004, less than 1% have been for fruit tree species. The development and testing of transgenic plum trees that are highly resistant to plum pox virus can serve as an example of the potential of genetic engineering for fruit tree improvement and also to illustrate the hurdles that this technology must overcome for the full realization of its potential in these species.

P-9

Can GE Trees Help the Environment? S. A. MERKLE. School of Forest Resources, University of Georgia, Athens, GA 30602. Email: smerkle@uga.edu

While the possible negative environmental impacts of the application of transgenic technology to forestry have been widely publicized, the potential positive environmental aspects of this technology have received much less attention. However, it is likely that some of the first applications of transgenic trees will be for the improvement of the environment and the conservation or restoration of forest trees. Transgenic technology may offer hope of conservation or restoration of forest species that have been devastated by insect pests or diseases to which no resistance has been found in the natural population. American chestnut (*Castanea dentata*) was wiped out as a canopy species by a fungal disease accidentally introduced into the United States around 1900. Similarly, American elm (*Ulmus americana*) has virtually disappeared as a favored street tree from Northeastern U.S. cities following the introduction of the Dutch elm disease fungus in the 1940s. Little or no natural resistance to these pathogens has been found in the native populations. While conventional breeding has made great progress in producing chestnut and elm trees that promise to be disease-resistant, an integrated approach that includes engineering with anti-fungal genes may accelerate the restoration of these "heritage trees." Because transgenic technology makes it possible to engineer trees with genes that simply do not exist in tree genomes, it offers the potential to adapt trees for production of goods and services never previously associated with forestry. One such service is environmental remediation. The use of biotechnology may make it possible to enormously enhance the abilities of plants, including forest trees, to take up and detoxify or sequester organic and heavy metal pollutants from soil and water. Cottonwood (*Populus* spp.) and sweetgum (*Liquidambar styraciflua*) trees engineered with genes to enhance breakdown of halogenated hydrocarbons or to detoxify mercury compounds are already being tested for their ability to remediate sites contaminated with these pollutants.

P-10

Regulation of Vegetative Phase Change in *Arabidopsis* by miRNAs and Endogenous Trans-acting siRNAs. SCOTT POETHIG. Department of Biology, University of Pennsylvania, Philadelphia, PA 19104. Email: spoethig@sas.upenn.edu

The transition between discrete stages of shoot development in plants is known as "phase change". At least two such stages exist during the vegetative phase of shoot growth—a juvenile phase and an adult phase. These phases are distinguished both by phase-specific vegetative traits and by the capacity of the shoot to undergo reproductive development. Mutations affecting the position of the juvenile-to-adult transition along the axis of the shoot have been identified in both maize and *Arabidopsis*. Cloning of these genes in *Arabidopsis* revealed that they encode proteins involved in the biogenesis or function of miRNAs and endogenous trans-acting siRNAs. Our studies demonstrate a role for post-transcriptional silencing (RNAi) in the regulation of vegetative phase change and have revealed the identity of some of heterochronic genes that are regulated by this mechanism.

P-11

Plant Growth Phases Affecting In Vitro Growth. SYLVIA ADJOA MITCHELL. University of the West Indies, Biotechnology Center, Mona, Kingston 7, St. Andrews 7, JAMAICA. Email: sylvia.mitchell@uwimona.edu.jm

Higher plants pass through a sequence of growth phases as they develop from a fertilized egg to an adult organism. In many plants these phases are distinguished by morphological differences. When explants are placed in the *in vitro* environment there is a general tendency towards rejuvenation, or an increase in juvenile characteristics and this is usually associated with an increased rooting and an increased multiplication rate. In *Dioscorea*, an herbaceous viny genus with both monocotyledonous and dicotyledonous characteristics, this general pattern was not found. For *Dioscorea*, whilst tissue culture did result in more juvenile-like growth (called type I) that had more profuse rooting than the adult-like form (type II), the juvenile-like form was slower growing. A single addition of a cytokinin (zeatin, 2-iP, BAP or kinetin) to the MS (1962) basal medium produced type II growth and an associated increased multiplication rate, the degree depending on yam species, hormone concentration and pH of the medium but not photoperiod. Addition of auxin (IBA, IAA, 2-4D or NAA), resulted in 100% type I growth. The effect of gibberellic acid has been extensively studied in *Hedera helix*, another viny species, and in this species GA₃ caused morphological reversion to the juvenile form; so the effect of GA₃ on two *Dioscorea* species was studied. The results were complex but indicated that gibberellic acid was able to negate to some degree the maturation effect of the added cytokinin with this effect being stronger for *D. trifida* than for *D. cayenensis*. By manipulation of the culture medium to maximize type II shoot growth, the number of sprouted minisetts after 31 months from one original node rose from 2,725 (when only type I growth produced) to 2,431,656 sprouted minisetts when type II growth was maximized. The implication of these results will be discussed.

P-12

Molecular Genetics of the AP3/PI Pathway in *Arabidopsis*. THOMAS JACK. Department of Biological Sciences, Dartmouth College, Hanover, NH 03755. Email: thomas.p.jack@dartmouth.edu

Angiosperm flowers consist of four organ types: sepals, petals, stamens, and carpels. We are interested in understanding the specification of organ identity in flowers of the plant *Arabidopsis thaliana*. Specifically, our studies focus on the floral organ identity genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*). In *ap3* and *pi* mutants, the petals are converted into sepals, and the stamens into carpels. *AP3* and *PI* are members of the MADS transcription factor family. We are presently pursuing both genetic and biochemical approaches to identify additional components of the *AP3/PI* pathway. Starting with the unusual *pi-5* allele, we carried out an enhancer/suppressor mutant screen. One mutant, *B class modifier 1* (*bcm1*) not only enhances the *pi-5* phenotype but also exhibits a phenotype in a wild-type background. Specifically, *bcm1* homozygous mutants develop filamentous structures in place of stamens. Via map-based cloning we are attempting to identify the *BCM1* gene. At present, we have strong evidence that *BCM1* encodes an AP2 domain protein. Our progress on the characterization of the role of this AP2 domain protein in petal and stamen development will be presented. In addition, our characterization of other *BCM* genes will be presented.

P-13

In Vitro Flowering in Orchids. K. RAJMOHAN. Kerala Agricultural University, Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala 695 522, INDIA. Email: rajmohan33@yahoo.com

Flowering is a complex process involving morphological and physiological changes, influenced by several external signals and internal factors. *In vitro* flowering is a powerful tool for plant physiologists and molecular biologists, to have better insight into the flowering process. In orchids, it has significant importance in hastening the development of new varieties. An exciting future in orchid breeding is predicted. There are a few reports on the *in vitro* flowering of orchids. We could induce *in vitro* flowering in the orchid *Dendrobium* var. Sonia 17, in a period of sixteen weeks. *In vitro* flowering is influenced by components of culture media and culture conditions. Roles of cytokinins like benzyl adenine, kinetin, thidiazuron, and zeatin; adenine sulphate; auxins like indole acetic acid and naphthalene acetic acid; gibberellic acid; abscisic acid; ethylene; interaction among plant growth substances; antagonists and inhibitors of plant growth substances, like paclobutrazol and silver nitrate; polyamines like spermidine and putrescine; hypomethylating agent like 5-azacytidine and regulating agents of osmotic potential, have been documented. Culture conditions like light, temperature and moisture content are important. Flower induction is influenced by the growth of other organs, nutrient status, carbon : nitrogen ratio, genotype, tissue type, sub-culturing, root excision etc. Treatments favouring rooting are detrimental for *in vitro* flowering. Various aspects of *in vitro* flowering in orchids will be presented.

P-14

The In Vitro Environment and Alterations to Growth. P. J. WEATHERS. Biology/Biotechnology Department, Worcester Polytechnic Institute, Worcester, MA 01609. Email: weathers@wpi.edu

The artificial environment created by *in vitro* culture can produce anomalies in growth and development. Besides the germplasm of the inoculum, the constituents found pre and post inoculum in the headspace gases and nutrient medium provide constantly varying conditions for impacting the cultures. For example, some strains of transformed root cultures can excrete auxins into their medium thereby complicating hormone addition studies. These same cultures can also produce ethylene, which if it accumulates in cultures, may alter not only growth, but also secondary metabolism. Further, the addition of sugars to the culture medium can dramatically affect not only growth and development of the roots, but also their secondary metabolism. In micropropagation, stale headspace gases and high humidity can induce hyperhydration and abnormal development of shoots and roots. To maintain normal growth and development in the *in vitro* environment, both gases and nutrient levels should be controlled. Whereas it is not so simple to control all of these conditions in all cases, there are methods that allow for both small and large scale control of culture conditions for improved culture growth. These approaches will be summarized here.

P-15

Availability and Balancing of Inorganic Nutrients in the Plant Tissue Culture Media. BYOUNG RYONG JEONG. Department of Horticulture, Gyeongsang National University, Jinju, KOREA 660-701. Email: brjeong@gsnu.ac.kr

The formulation of the culture medium is important in developing plant tissue culture/micropropagation protocols. In plant tissue culture the medium volume is usually relatively small (4~10 mL per plantlet) as compared to that in hydroponics and other plant production systems. In addition, the tissue culture medium is not replenished for a long period of time, making the initial medium composition critically important. An ideal tissue culture medium should provide plantlets with sufficient amounts of essential nutrients to the end of culture so that such nutrients do not limit growth. On the other hand, the medium should contain the minimum possible amounts of nutrients to minimize the water- and chemical-stresses to plantlets, especially at the early stages of culture. Hence, varying nutrient contents in the culture medium is one of the ways to regulate the growth of the plantlet. Due to lack of a convenient nutrient resupplying method, plant tissue culture medium must be supplied with a complete nutrient formulation. Many researchers in plant tissue culture area often experience difficulty in adjusting/balancing inorganic nutrients, especially when their water contains minerals, carbonate and bicarbonate in excess amounts. A system of balancing anions and cations according to water analysis has been developed and has been used widely in hydroponics. Water analysis expressed in me/L ionic concentrations allows a flexibility in salt/fertilizer selection and adjustment. Nutrient requirements of selected plant species and examples of ion balancing for plant tissue culture medium will be presented.

P-16

Assessing the Bioavailability of Enhanced Dietary Carotenoids in Vegetable Crops. D. A. KOPSELL and Joanne Curran-Celentano. ¹Department of Plant Sciences, The University of Tennessee, Knoxville, TN 37996 and ²Department of Animal and Nutritional Sciences, The University of New Hampshire, Durham, NH 03824. Email: dkopsell@utk.edu

Phytonutrient enhancement of vegetable crops is an emerging area in plant physiology. One area gaining attention is the role of diet in the eye health of older adults. Age-related macular degeneration is the loss of vision in the central region of the sight pattern, increasing in incidence with the aging population. Lutein and beta-carotene are two plant-derived, dietary carotenoids responsible for antioxidant and photoprotective functions in the macular region of the eye. The goal of our research program is to investigate the ability to enhance the carotenoid content of *Brassica* and *Spinacia* vegetable crops. Our project objectives were: 1) to determine how environmental and genetic factors affect the concentration of carotenoid compounds in vegetable crops; and 2) to assess the bioavailability of dietary carotenoids after consumption of vegetables vs. mono-molecular supplements. Research compared blood serum carotenoid profiles and macular pigment optical densities (MPOD) in human subjects administered various dosages of mono-molecular lutein supplements, received from commercial sources, or lutein enhanced spinach products, grown at the Woodman Horticulture Farm (Durham, NH). Treatments included a placebo supplement group, a 10 mg lutein supplement group, a 30 mg lutein supplement group, a 9 mg lutein spinach group, and a 12 mg lutein spinach group. Heterochromatic flicker photometry (HFP) was used to determine MPOD at 4 separate retinal loci. There were increases in MPOD at all the retinal loci (20', 30' and 60') in subjects receiving spinach with 12 mg lutein and the 30 mg lutein supplement. This data supports the importance of enhancement efforts to increase valuable dietary carotenoids.

P-17

Bioavailable Iron - The Determinant in the Iron Nutritional Value of Food Crops. R. M. WELCH. USDA-ARS, U.S. Plant, Soil & Nutrition Laboratory, Cornell University, Ithaca, NY 14853. Email: rmw1@cornell.edu

Over 3 billion people globally are iron deficient mostly among the poor in the Global South. Commonly, total iron consumption from typical diets is enough to meet nutritional needs. However, many people dependent on staple plant foods as their major source of iron do not consume enough bioavailable iron to meet their nutritional requirements for iron. This results from the fact that most staple food crops (e.g., legume seeds and cereal grains), as eaten, contain antinutritive substances (e.g., phytate, polyphenols, etc.) that inhibit the absorption of iron from the gut. Thus, bioavailable iron levels in food crops and diets are an important factor to consider when addressing iron deficiency among vulnerable populations. Various models have been developed to test iron bioavailability in foods and diets. We have employed an *in vitro* model using human Caco-2 cells that allows rapid screening of large numbers of staple food crops and diet constituents for bioavailable iron. This model has also been used in studies directed at isolating and identifying those factors that either promote or inhibit iron bioavailability from foods and complex diets. It can also be used in molecular biology experiments directed at improving the nutritional value of iron in transformants. Examples of the use of this model in these types of studies will be discussed.

P-18

Will Flowers Smell Again? N. DUDAREVA. Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907. Email: dudareva@purdue.edu

In nature, structure, color, and scent are critical factors in attracting pollinators to flowers. The same features determine consumers' attraction to cut flowers, potted flowering plants, and flowering herbaceous plants, which play an important role in human life from an esthetic point of view. Floral scent is a diverse blend of low molecular weight (under 300 Da) mostly lipophilic compounds emitted from flowers into the surrounding atmosphere. While the chemistry of plant volatiles is well understood, less is known about the biosynthesis of this diverse group of compounds. Using functional genomic and biochemical approaches we have identified and characterized several genes responsible for the formation of scent volatiles. Diurnally emitting snapdragon and nocturnally emitting petunia were used as model systems to study the flux through the metabolic pathway(s) *in situ*. We found that volatiles are synthesized *de novo* in the epidermal cells of plant tissues from which they are emitted and their production and emission is spatially and developmentally regulated. The amount and/or type of emitted compound is often determined by the availability of substrates for the final reaction, especially for enzymes with broad substrate specificity. While petunia flowers produce almost exclusively benzenoid/phenylpropanoid compounds, snapdragon floral scent is rich in both terpenoids and phenylpropanoids. In snapdragon the orchestrated emission of volatiles produced by more than one biochemical pathway is regulated upstream of individual metabolic pathways and includes the coordinated expression of genes that encode enzymes involved in the final steps of scent biosynthesis. Recent advances in the isolation of scent biosynthetic genes have opened up the possibility for the genetic manipulation of floral scent, which was only partially successful. A comprehensive understanding of the regulation of floral scent production in plants will provide a foundation for successful metabolic engineering of the odorant trait.

P-19

Molecular Growth Regulation of Warm Season Turfgrass. F. ALTPETER, M. Agharkar, H. Zhang. University of Florida - IFAS, Agronomy Department, PMCB, Laboratory of Molecular Plant Physiology, 2191 McCarty Hall, P.O. Box 110300, Gainesville, FL 32611-0300. Email: faltpeter@ifas.ufl.edu

Bahiagrass (*Paspalum notatum* Flugge) is a low input, drought tolerant and disease resistant warm season turfgrass used for residential lawns and along highways in the Southeastern US. However turf quality of currently available bahiagrass cultivars is poor, due to prolific seedhead production, open growth habit and light green color. The objective of this experiment was to develop a genetic transformation protocol for bahiagrass and over-express a gibberellin catabolizing enzyme, Gibberellin 2-oxidase, to enhance turf quality and reduce mowing frequency. Gibberellin 2-oxidase cDNA was isolated from *Arabidopsis* using primers as suggested by Schomburg et al. (2003). Co-transfer of a constitutive *nptII* (Altpeter et al. 2000) and GA-2 oxidase expression cassette, into seed derived callus cultures from turf-type bahiagrass (cv. 'Argentine') was followed by selection with paromomycin sulphate during callus subculture and, or regeneration (Altpeter and James 2005). Transgenic plants were confirmed by NPTII ELISA (Agdia), PCR and altered phenotype. **Conclusions:** Over-expression of Gibberellin 2-oxidase from *Arabidopsis* in bahiagrass resulted in dwarf phenotypes. Transgenic plants were established successfully in soil. Data correlating Gibberellin 2-oxidase over-expression in transgenic bahiagrass with physiological parameters will be collected. **References:** Altpeter, F., J. Xu, & S. Ahmed. (2000). Generation of large numbers of independently transformed fertile perennial ryegrass (*Lolium perenne* L.) plants of forage- and turf-type cultivars. *Mol. Breeding* 6:519-528. Altpeter, F. & V. James (2005) Genetic transformation of turf-type bahiagrass (*Paspalum notatum* Flugge) by biolistic gene transfer. *Intern. Turfgrass Soc. Res. J.* (accepted for publication). Schomburg, F. M., C. M. Bizzell, D. J. Lee, J. A. D. Zeevaart & R. M. Amasino (2003) Overexpression of a novel class of Gibberellin 2-Oxidases Decreases Gibberellin Levels and Creates Dwarf Plants. *Plant Cell* 15:151-163.

P-20

Plant-based Vaccines: Prospects After 15 Years of Research. H. S. MASON. Biodesign Institute at Arizona State University, Tempe, AZ 85287-5401. Email: Hugh.Mason@asu.edu

After the first patent on plant-based vaccines was published in 1990, and the first peer-reviewed publication in 1992, there was first a gradual and then exponential increase in the work done in this area. There are now many demonstrations of production of vaccine antigens in plants, and fewer but substantial numbers of studies showing antibody responses in animals that were administered the plant-derived vaccines. However, there are very few studies that show true vaccine activity, i.e., protection from disease upon challenge with the causative agent, and no plant-based vaccine has been registered commercially. The reasons for this are many, including limited coordination between plant scientists and medical or veterinary scientists, relatively poor antigen expression in many plant systems, a bias toward oral delivery of crude plant preparations, and the inherently poor immunogenicity of most subunit antigens in the gut. Moreover, most vaccine development is performed by pharmaceutical companies, and these entities are rightly reluctant to invest the funds needed on a system whose commercial potential is uncertain due to murky issues of industry regulation and intellectual property. The prospects for addressing these problems and the realization of plant-based vaccines will be discussed.

P-21

Plant-based Technologies: Potential for Vaccine Development and Delivery. Konstantin Musiychuk, Natalia Ugulava, Vadim Mett, and VIDADI YUSIBOV. Fraunhofer USA Center for Molecular Biotechnology, 9 Innovation Way, Suite 200, Newark, DE 19711. Email: vyusibov@fraunhofer.org

Interest in plants as an alternative system for producing therapeutic proteins and vaccine antigens is rapidly increasing. This approach provides an experimental platform for the time-efficient production of candidate vaccines against single or multiple pathogens. One of the more recent developments is the use of plant RNA viruses as expression vectors for the production and delivery of antigenic determinants. To this date a number of plant RNA viruses such as, *Tobacco mosaic virus*, *Cowpea mosaic virus*, *Cucumber mosaic virus*, *Tomato bushy stunt virus* and *Alfalfa mosaic virus* have been successfully used to express and deliver antigenic determinants from different pathogens. At Fraunhofer USA CMB we use plant virus-based expression vectors to produce target proteins. One of our strategies for expression, recovery and delivery of antigens is based on using a thermostable enzyme (maintains full activity at 75° C), beta-1,3-1,4-glucanase (LicB), from *Clostridium thermocellum* as a carrier for target polypeptides of up to 100 kDa. The polypeptides can be produced as N-, C-terminal or internal fusions in the surface loop of the protein. Recombinant LicB can be efficiently produced in variety of expression systems, including plants. The thermostable properties of LicB facilitate purification of the candidate antigens. We have used this approach to engineer and produce more than ten target antigens from different pathogens, including, *Respiratory syncytial virus*, *Human immunodeficiency virus* and *B. anthracis* [Domain four (145 amino acids) of PA and N-terminus (220 amino acids) of LF of anthrax toxin]. A recombinant LicB that contained Domain four of PA of *B. anthracis* (LicB-PAD4) had specific reactivity to polyclonal rabbit sera from animals immunized with PA, human sera from individuals that received AVA vaccine and monoclonal antibody 14B7 that specifically reacts with Domain four, indicating that the conformation of Domain four was maintained in the fusion protein. Mice immunized with three doses of recombinant LicB-PAD4 responded by mounting strong PA-specific IgG responses, and sera from these animals demonstrated neutralizing activity against Lethal Toxin from *B. anthracis*.

P-22

Corn-based Vaccines: Challenges and Opportunities. K. WANG¹, S. Karaman¹, and J. Cunick². ¹Center for Plant Transformation, BioPharmaceuticals Initiative, Plant Science Institute, Iowa State University, Ames, IA 50011-1010 and ²Department of Animal Sciences, BioPharmaceuticals Initiative, Plant Science Institute, Iowa University, Ames, IA 50011. Email: kanwang@iastate.edu

LT-B is a non-toxic subunit of a holotoxin (hLT), which causes diarrhea, produced by enterotoxigenic strains of *E. coli*. It is highly immunogenic and can be a potent adjuvant for co-administered antigens. We have previously demonstrated that a strong mucosal and systemic immune response in young mice could be elicited upon administration of corn-derived LT-B in the form of feed pellets. Orally immunized mice were protected against hLT and CT (cholera toxin) challenge (Chikwamba et al., *Transgenic Research* 11: 479, 2002). In addition, using immunogold labeling/electron microscopy, cell fractionation and protein analysis techniques, we observed that LT-B protein could be detected both internally and externally on starch granules of corn kernels (Chikwamba et al., *PNAS* 100: 11127, 2003). We further investigate long-term systemic and mucosal immune responses and secondary response in aged mice that were orally administered corn-derived LT-B when they were young. Systemic and mucosal antibody levels were monitored for 11 months in mice that had been immunized with LT-B corn at 3-month of age. Our results indicated that anti-LT-B secretory IgA and IgG antibodies are detectable over 14-month period even though the level of antibodies declines gradually. However, booster feeding and injections dramatically increased the immunoglobulin levels that had declined over the previous 11 months after initial feedings. These data indicate that corn-derived LT-B can elicit systemic and mucosal immune responses that persist for long time. In addition, re-administration of the antigen through oral and parenteral routes in aged mice can effectively enhance the immune response against the antigen. We have conducted small scale field release for seed increase purpose for the LT-B corn in confined field conditions in both Iowa and Colorado for three summer seasons. Preliminary analysis on inadvertent occurrence of corn-based pharmaceuticals in food supply and its impact on human health will be presented.

P-23

The Vitamin C Network - New Branches in Plant Biochemistry. C. L. NESSELER, A. Lorence, B. Chevone, and P. Mendes*. Department of Plant Pathology/Virginia Bioinformatics Institute* Virginia Tech, Blacksburg, VA 24061. Email: cnessler@vt.edu

L-Ascorbate is a major antioxidant molecule, an essential cofactor for several important metal-containing enzymes and is implicated in control of cell division and growth. Understanding plant AsA biosynthesis is important because they are the major dietary source of vitamin C for humans. The pathway that leads to AsA in animals has been known since the 1950s. However, it was only in the late 1990s that a plant model was proposed that explains radiolabel feeding results. This model, known as the Smirnoff-Wheeler pathway, proposes D-mannose (Man) and L-galactose (Gal) as the main intermediates for AsA biosynthesis. Recently different research groups, including ours, have obtained evidence for additional AsA biosynthetic pathways in plants. The new entry points for this network are: D-galacturonic acid, and myo-inositol (MI). Only a few genes encoding enzymes from each branch of the network have been cloned and characterized. We have data supporting the importance of the MI pathway for AsA biosynthesis and for normal growth and development. The first enzyme in the MI pathway is MI oxygenase (MIOX, EC 1.13.99.1). We have found that the MIOX family is composed of four members in the *Arabidopsis thaliana* genome: *miox1*, *miox2*, *miox4* and *miox5*, numbered according to the chromosome on which they are located. Assays performed with homozygous T-DNA knockout lines revealed that *miox1* is the family member that contributes the most to the AsA content of Arabidopsis leaf tissue. The next enzyme in the MI pathway catalyzes the conversion of the MIOX product, D-glucuronic acid (GlcUA) to L-gulonic acid. This enzyme known as GlcUA reductase (EC 1.1.1.19), belongs to the family of aldo/keto reductases. We identified close to 40 members of this family in the *Arabidopsis* genome. Screening of T-DNA knockout lines using HPLC and spectrophotometric-based assays of some of the members of the aldo/keto reductases allowed us to identify three SALK lines which had a substantial reduction (~50%) of AsA in rosette leaves compared to wild type. Expression of these ORFs in wt plants leads to a two-fold increase in foliar AsA.

P-24

Inducible Polyhydroxybutyrate Formation in *Arabidopsis*. LAURALYNN KOURTZ, Kevin Dillon, Sean Daughtry, Oliver Peoples, and Kristi D. Snell. Metabolix, 21 Erie Street, Cambridge, MA 02139. Email: lkourtz@metabolix.com

Polyhydroxyalkanoates (PHAs) are a family of biodegradable biopolymers capable of matching or exceeding the performance characteristics of petroleum-based plastics. PHB, a short-chain member of the PHA family, is currently being produced via large-scale *Escherichia coli* fermentations, but the development of agricultural systems, in which bioplastics can be produced economically and sustainably in green plants, has the potential to not only dramatically lower the cost of the bioplastics, but to sequester CO₂. The production of polyhydroxybutyrate (PHB), which involves a multigene pathway consisting of a thiolase, a reductase and a synthase gene, has been achieved in several plant species, but high levels of polymer production in chloroplasts resulted in chlorotic plants that failed to achieve full size. The use of inducible promoter systems and their potential to surmount this barrier to large-scale PHB production by permitting plants to achieve full-size growth before turning on the PHB production machinery will be discussed. In addition, the ability of gene fusion to simplify the PHB biosynthetic pathway for plant based expression will be addressed.

P-25

Production of Gums in Plants. K. S. DHUGGA. Crop Genetics Research and Development, Pioneer Hi-Bred International, Inc., A DuPont Company, Johnston, IA 50131. Email: kanwarpal.dhugga@pioneer.com

Gums are high molecular mass polysaccharides that can absorb large volumes of water, resulting in viscous solutions. Their ability to alter the rheological properties of solutions makes them suitable for many industrial uses. Although most commonly used as food additives to provide stiffness and texture, prevent ice crystal formation, maintain crispiness, and retain moisture, gums have many other applications in the non-food industries. For example, they are used in: textile as dyeing and printing aids; cosmetics to thicken shampoos and conditioners; paper as binders and hardeners; construction to alter flow properties of concrete; petroleum as drilling agents for oil and gas wells; mining and mineral for separation of minerals from crude ores; and explosives to thicken slurries and as desiccants. The seeds of some plants accumulate hemicellulosic polysaccharides as storage carbohydrates, a role that is analogous to that of starch in cereals. For example, guar and locust bean seeds accumulate galactomannan, the most commonly used plant gum, in their endosperm. We have isolated the gene that forms mannan, the backbone of galactomannan, from guar through a combination of genomic and biochemical approaches. This has allowed us to employ biotechnological approaches to exploit the synthesis of this polysaccharide in a commercial crop, soybean. Initial results are encouraging in that the soybean seeds expressing the guar mannan synthase gene contain elevated levels of mannose. Ability to produce galactomannan in non-native species has opened the door for producing high quality as well as novel gums.

P-26

Response of Soybean Cell Cultures to Biological Elicitors for the Production of Antiestrogenic Phytoalexins. S. M. BOUE¹, S. Kale-Ireland*, S., Khan², K. Rajasekaran¹, B. Shih¹, C. Carter-Wientjes¹, J. McLachlan², and T. E. Cleveland¹. ¹SRRC, USDA, New Orleans, LA 70124 and ²Tulane-Xavier CBR, New Orleans, LA 70112. Email: sboue@src.ars.usda.gov

Soybean products are known for their high protein content and their production of isoflavones (natural products) which have estrogenic activity. Interestingly, soy plants also display a natural resistance to *Aspergillus flavus* and *A. parasiticus*, the fungi that make the highly mutagenic and carcinogenic secondary metabolites, the aflatoxins. Since the aflatoxigenic fungi routinely infect crops of human and animal consumption, but the soybean plant is able to avoid this infection, it is possible that the soybean cells release stress-response compounds, mainly isoflavones called phytoalexins, which offer a protective role to avoiding this infection. Some of these phytoalexins, including genistein and daidzein, have been shown to be beneficial to human health. In this study the ability of several elicitors at inducing phytoalexins in soybean liquid cell culture were evaluated. Of particular interest was the ability of each elicitor to induce the isoflavone phytoalexin glyceollin, which has recently been shown to be antiestrogenic in several *in vitro* assays. Soybean cells from both control and elicitor-exposed cultures were analyzed using HPLC-MS. Several constitutive isoflavones have been identified and preliminary data indicate novel phytoalexins related to the glyceollins are formed.

W-1

Perfusion of Organs. S. BAICU. Cell and Tissue Systems Inc., Charleston, SC 29403. Email: sbaicu@celltissuesystems.com

To balance the organ supply-demand and successfully select and use good quality organs from expanded criteria and non-heart-beating donors, efficient preservation and viability assessment methods are needed. Machine perfusion has been efficiently used for hypothermic perfusion of kidneys. Flow rate, vascular resistance, and biopsy histopathology have been the only accepted indicators of kidney viability prior to transplantation. Perfusion solution chemical composition plays a critical role in the organ transplantation outcome, and has a strong effect on renal biochemistry and pH dynamics. Solutions characterized by high buffering strength provide tighter pH control during *ex-vivo* kidney preservation. Kidneys with similar pump characteristics (i.e., flow rate and vascular resistance) exhibit different metabolic activity (perfusate concentration dynamics of electrolytes and metabolites) when perfused with solutions of various chemical formulations. Monitoring renal biochemistry directly at the cellular sites of metabolic and synthetic activity, at the interstitial level not through the biochemical analysis of renal effluent can provide accurate information regarding the *ex-vivo* functional status of kidneys.

W-2

Towards the Development of a Pancreatic Tissue Substitute: Preservation of Encapsulated Cell Systems. A. SAMBANIS^{1,2,3}, N. Mukherjee^{1,2}, Z. Chen⁴, and Y. Song⁴. ¹School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA 30332; ²Georgia Tech/Emory Center for the Engineering of Living Tissues, Georgia Institute of Technology, Atlanta, GA 30332; ³Georgia Tech/Emory Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA 30332; and ⁴Organ Recovery Systems, Inc., Charleston, SC 29403. Email: Athanassios.Sambanis@chbe.gatech.edu

Cell-based therapies involving implantation of glucose-responsive, insulin-secreting cells offer significant promise in providing a more physiologic, less invasive, and potentially less costly treatment for insulin-dependent diabetes than daily insulin injections. With allo- or xenogeneic cells, cells are commonly encapsulated in semi-permeable membranes for partial protection from the immune system of the host. The membrane pores allow passage of low molecular weight nutrients and metabolites, including insulin, but exclude antibodies and cytotoxic cells of the host. With autologous cells, such as non-pancreatic cells retrieved as a biopsy and engineered for insulin secretion, encapsulation may still be needed to better sustain cell function, localize the implanted cells, and make the implant retrievable. In both cases, long-term preservation of cells and/or constructs is essential for off-the-shelf product availability. One possible route to address this is through cryopreservation. With encapsulated cell system, all three components of the capsule, namely cells, matrix and semi-permeable membrane, need to maintain their functionality post-preservation for a procedure to be successful. This presentation describes the critical issues in cell-based therapies and encapsulated cell systems for treatment of insulin-dependent diabetes; challenges in cell and construct preservation; and results with mouse insulinoma cells encapsulated in calcium alginate/ poly-L-lysine/ alginate beads and subjected to either conventional freezing or ice-free cryopreservation (vitrification). The importance of considering construct preservation early in the design of tissue substitutes and encapsulated systems in particular will be discussed.

W-3

In Vitro Models of Liver Steatosis and Ischemia/Reperfusion. KENNETH D. CHAVIN. Department of Surgery, Division of Transplantation, Medical University of South Carolina, Charleston, SC 29425. Email: chavinkd@musc.edu

The quality and quantity of livers available for transplantation continues to decline with the rise in obesity, which is associated with fatty liver (steatosis). The degree of liver graft steatosis directly correlates with injury and failure following transplantation. Steatosis is a primary determinant for the use of a graft for transplantation. Many animal models exist which are used to determine the mechanisms associated with liver graft function. However, sophisticated in vitro models are necessary for understanding the predisposition of steatotic livers/hepatocytes to ischemia/reperfusion (I/R) injury. To investigate these conditions, our lab has developed two in vitro models using Hep 6-16 cells and isolated steatotic hepatocytes. Using Hep 6-16 cells, we have successfully simulated an ischemic state through inhibition of oxidative phosphorylation with FCCP, and have mimicked the steatotic phenotype through forced expression of uncoupling protein-2 (UCP2). We previously reported that UCP2 expression in steatotic livers is increased, which is associated with depleted hepatic ATP stores and renders steatotic hepatocytes vulnerable to ischemic damage. Using our models, we have determined the function of UCP2 and its contribution to the development of I/R injury. Furthermore, they have allowed us to explore the differences in mitochondrial energy state between lean and steatotic hepatocytes and how this contributes to proliferation, regeneration and resistance to I/R injury. Ultimately, by better understanding the biochemistry of fatty livers and hepatocytes, we plan to design therapies which will make steatotic livers more usable in the human transplant setting.

W-4

Challenges and Solutions for Biopreservation of Cells, Tissues, and Organs. A. J. MATHEW¹, J. M. Baust², K. K. Snyder², R. G. Van Buskirk², and J. G. Baust¹. ¹BioLife Solutions and ²Cell Preservation Services, Owego, NY 13827. Email: amathew@biolifesolutions.com

With the recent explosion in the development of complex biologics for utilization in reparative/ regenerative medicine (cell therapy, tissue engineering), the need for biopreservation has become critical. Due to the diversity of post-preservation responses of individual systems, traditional methodologies have resulted in a "bottleneck" in application to new therapeutic technologies thereby limiting banking and distribution. In an effort to overcome this, we have begun investigation of the molecular responses of biologics to the preservation process (hypothermic storage and cryopreservation) in order to understand the mechanisms of traditional preservation failure or limitation. These efforts have identified diverse molecular-based responses to preservation and facilitated the development of new technologies for the improvement of both viability and function post-storage. These new technologies also provide increased productivity, improved time management, and enhanced distribution capabilities for cell, tissue and organ systems. This seminar will provide a discussion of the fundamentals and recent advances in biopreservation which are proving critical for the processing of biotherapeutic cells and tissues, as well as systems being banked and utilized in pharmaceutical, drug discovery, and basic sciences arenas. Regulatory issues and challenges, such as identifying the appropriate solution and inclusion of preservation solutions into IND's, will also be addressed.

Workshops

W-5

The In Vitro Collecting Kit. V. C. PENCE. Center for Conservation and Research of Endangered Wildlife (CREW), Cincinnati Zoo & Botanical Garden, 3400 Vine Street, Cincinnati, OH 45220. Email: valerie.pence@cincinnati-zoo.org

The In Vitro Collecting (IVC) Kit was developed at CREW to provide teachers and students with a hands-on activity involving plant tissue culture and information on plant conservation. IVC is the process of initiating plant tissue cultures in the field and is a technique that has been a focus of research at CREW, where it is used to collect tissues from endangered plants for propagation. Because IVC is done outside of the laboratory without the benefit of a laminar flow hood, it is well suited for classroom activities. The kit's manual describes four experiments that illustrate the challenges of contamination and the effects of media on plant tissue cultures. Students collect small pieces of plant tissues, surface sterilize them, and place them into small vials of media containing different antimicrobial agents and different hormones. The kit contains all of the media, tools, and supplies needed for the experiments, except the plant. Students learn about sterile technique, surface and internal contaminants, and the effects of auxins and cytokinins in plant growth in culture. In addition, there is background information on plant conservation and endangered species preservation. The kits are part of a developing collection of *Junior Plant Scientist* modules that are designed to provide science-based activities related to plant conservation to older elementary, junior high, and high school students. They can be used in the classroom or individually, e.g., as science fair projects. The kits are assembled by CREW staff and volunteers, and the proceeds from their sales support the Endangered Plant Propagation Program at CREW.

W-6

Tissue Culture Kits from PhytoTechnology Laboratories. G. R. SECKINGER and K. C. Torres. PhytoTechnology Laboratories, 14335 West 97th Terrace, Lenexa, KS 66215. Email: info@phytotechlab.com

PhytoTechnology Labs has introduced several kits for both hobby and classroom applications. They include: four kits for the multiplication of African violets, Boston ferns, Hostas, or Lilies; two kits for the sowing of epiphytic or terrestrial orchid seeds; and an orchid stem (e.g., *Phalaenopsis*) propagation kit. These kits are designed to provide the basic media and supplies necessary to demonstrate micropropagation stages I-III while being flexible to permit additional experimentation. Certain kits also include established cultures which can be subcultured for rapid stage II demonstration. Each kit includes instructions to prepare and dispense media, surface sterilize plant tissue, initiate and maintain cultures, and establish plants *ex vitro*. The instructions also provide a list of kit components, media formulations, and a list of items needed but not included with the kit, e.g., beakers, distilled water, alcohol, bleach. The scope of the kit or number of users can be expanded as additional kit components may be purchased separately.

W-7

Kits from Carolina Biological, Ward's Scientific, and Kitchen Culture Kits. C. M. STIFF. Kitchen Culture Kits, Inc., 905 Champions, Lufkin, TX 75901. Email: kck@turbonet.com

Several commercial plant tissue culture kits have been developed over the years for use in the classroom by Carolina Biological and Ward's Scientific among other companies. Another kit has been developed primarily for hobbyists by Kitchen Culture Kits. These have all been developed without need for a laminar flow hood thus making them affordable for use in the classroom. We will discuss the various kits: the advantages and disadvantages of each for the classroom, experiments that provide hands-on experience for the students, and how they can be utilized for classroom and individual projects.

W-8

Jamaican 'Do-it-yourself' Tissue Culture Kit. SYLVIA MITCHELL, Kimberly Wray, and M. H. Ahmad. Biotechnology Center, University of the West Indies, Kingston, JAMAICA. Email: sylvia.mitchell@uwimona.edu.jm

Tissue culture was introduced into Jamaica in the 1960s and quickly became used commercially for the propagation of orchids. By 2004, many years later, orchids still remain the only crop micropropagated by the private sector in Jamaica although there are a few public labs that multiply small numbers of elite plants by tissue culture. To encourage innovation and to get tissue culture more integrated into the educational, and agricultural and horticultural propagation sector, low-cost tissue kits are being devised. Kits have been produced where costs were reduced in four main areas without compromising the quantity or quality of the plantlets: the type of aseptic transfer area used, nutrient media, culture incubation and sterilization equipment. A Mobile Micropropagation Unit (MMU) was created as a laminar flow substitute: 83% of leaf of life cultures, *Bryophyllum pinnatum*, remained clean after initiation compared to 88% of those transferred under the conventional laminar flow cabinet. Hormone supplemented MS (1962) media produced shoots, roots or callus from leaf explants within three weeks. This plant, MMU and media have successfully been used to teach tissue culture in both undergraduate and postgraduate biotechnology courses at the University of the West Indies, Jamaica. A nutrient media of local origin (using liquid fertilizer [Peter's 20-20-20] and rain water without any phytohormones or micro-nutrients) gave average shoot and root numbers of 4.1 and 16.6 four weeks after initiation respectively. A local gelling agent has also been successfully used. It is planned to test these kits in primary, high schools and teacher's colleges to further refine these kits before releasing them to the market.

W-9

Global RNAi Phenotype Analysis for Cancer Drug Target Identification. David Azorsa, David Evans, Jeff Kiefer, Qiang Que, Tom McCarty, Hong Wang, Haiyong Han, Sukru Tuzmen, Michael Bittner, Olli Kallioniemi, Jeffrey Trent, Daniel Von Hoff, and SPYRO MOUSSES. Cancer Drug Development Laboratory, Translational Genomics Research Institute (TGen), Gaithersburg, MD 20878. Headquarters: Phoenix AZ. Email: smousses@tgen.org

Gene regulatory networks that control growth and survival of cancer cells are affected by many intrinsic and extrinsic factors. The molecular and genetic context of a cell defines a particular state of a cell and also determines the relative dependency on certain genes that are essential for growth and survival. To better understand the onco-selective dependencies that arise during the neoplastic process, we have developed and applied global RNAi profiling to discover context dependent vulnerabilities in cancer cells. Specifically, we have used high throughput transfection of 10,000 siRNA, (individually targeting 5000 druggable gene targets) to systematically knock down 5000 genes in parallel, and analyzed the effects on growth and survival in human cancer cells. Multidimensional analysis of various cancer cell line models enabled the identification of genes which were selectively and differentially required for growth. When comparing global RNAi profiles across multiple cell lines with very different genetic backgrounds, we were able to identify gene knockdowns that were very selective and cell line specific in their phenotype. To understand the source of selectivity, we began by integrating the RNAi phenotype data with gene expression and gene copy number data from microarray analysis. This revealed that some of these contextual vulnerabilities were associated with DNA amplification, suggesting that they may be etiologically relevant dependencies, but others appeared to be simply dependent on cell type. To gain a deeper understanding on how specific cancer associated perturbations can alter the relative dependency on specific genes and pathways for survival, we created isogenic cell lines that vary only in the expression of a single tumor suppressor gene. Using these model cell lines, we were able to identify synthetic lethal RNAi targeting events that selectively killed cancer specific genetic defect. Many of these contextually vulnerable targets are currently being advanced towards traditional drug discovery to develop novel anticancer agents for patients with defined genetic alterations in their tumors. Finally, we exposed cancer cell lines to various anticancer drugs to model the cellular context of drug response, and screened for RNAi targeting events that appeared to enhance or suppress drug response. Using a functional pharmacogenomic screen for genes that affect cancer chemotherapy response, we were able to identify functionally relevant genes that could give us a better understanding of mechanism of drug action, functionally relevant candidate markers for predicting drug response, and candidate drug targets for combination therapy to enhance the response to common cancer drugs.

W-10

From Basics to Practice—Methodologies and Applications of Highly Effective and Specific RNAi. K. E. LOWRIE, P. Welch, M. Bunting, K. Wiederholt, and S. Hough. Invitrogen Corporation, Carlsbad, CA 92008. Email kerry.lowrie@invitrogen.com

RNA interference (RNAi) is an evolutionarily conserved cellular process that has become a powerful new research tool to mediate sequence-specific gene silencing in a wide variety of mammalian cell types and whole organisms. In this pathway, a short dsRNA duplex is believed to assemble with a series of proteins, referred to as the RNA-induced silencing complex (RISC), that guides hybridization of the duplex antisense strand to its complementary target sequence and initiates cleavage of the target mRNA. RNAi has now replaced more time consuming and less robust technologies such as antisense, ribozymes, and homologous recombination as the principle approach for loss of function studies. However, despite the effectiveness of RNAi to inhibit the expression of select genes, it is important to be aware of the limitations and potential for undesired effects that can lead to the erroneous interpretation of RNAi experiments. To achieve the highest quality RNAi results requires selection of the most appropriate RNAi reagents and controls, combined with robust cellular delivery and high specificity of target knockdown. The first part of this presentation will review the history and mechanism of RNAi and discuss experimental considerations and controls that are important to successful experiments. Gene silencing can be achieved through the introduction of short interfering dsRNA (siRNA), chemically modified duplexes such as Stealth[®] RNAi, or short hairpin RNA (shRNA) expression vectors. The second half will describe tools for design and selection of the most appropriate RNAi reagents based on the experimental approach, and will describe RNAi applications to functionally dissect cellular pathways and stimulate cell differentiation.

W-11

Practical and Regulatory Considerations in the Development of Mammalian Cell-based Biopharmaceuticals. W. ALAN MOORE. Althea Technologies, Inc., 3550 General Atomics Court, Building 2, San Diego, CA 92121. Email: amoore@altheatech.com

This session will explore the use of mammalian cells in the discovery and development of biopharmaceutical products for therapeutic use as regulated products of drugs, biologics, including biological devices and cellular therapies, or medical devices with biological components. Attention will be focused on pragmatic solutions for the early screening and advancement of candidates, recognition of significant regulatory considerations at the earliest stages of development and practical considerations in the early-stage manufacture of product for pre-clinical and clinical trial evaluation.

W-12

Nucleofection[®]: A Novel Non-viral transfection Method for Primary Cells and Hard-to-transfect Cell Lines. T. KRETZSCHMAR. amaxa GmbH, Köln, GERMANY. Email: titus.kretzschmar@amaxa.com

amaxa is dedicated to developing high-quality tools for the non-viral gene transfer. Nucleofection is an easy-to-perform transfection method particularly well suited for engineering of primary cells as well as other so-called “hard-to-transfect” cell types. The technology is based on unique combinations of specific electric pulses and cell solutions that have been optimized for a great variety of cell types. Applying nucleofection, genetic material is not only delivered to the cytosol but also directly to the cell nucleus. Hence, even non- or slowly proliferating cells are transfected at high numbers with the benefit of short time intervals between nucleofection and subsequent analyses. Latest news of the nucleofection of a very large diversity of cells relevant for research, development, as well as therapeutic purposes will be presented including data on efficiencies, viabilities, and functionalities.

W-13

Considerations When Using Lipid Based Transfection Reagents. LINDA B JACOBSEN¹, and Manfred Watzel². ¹Roche Applied Science, Indianapolis, IN 46256 and ²Penzberg, GERMANY. Email: Linda.Jacobsen@roche.com

Although many lipid based transfection reagents are easy to use, there are still several procedural steps that scientists can modify to optimize their results. These include: optimization of the reagent:DNA ratio, optimization of the total amount of complex added, passage level of cells, plating density of cells, diluent for complex formation, incubation time of complex, and time of exposure of complex to cells. Varying these parameters can maximize protein expression, and minimize cytotoxic effects. Additionally, it is important to understand that transfection may affect many other genes in the cells. Different methods for evaluation of expression can produce conflicting results. These methods include % cells transfected, average intensity of expression per cell, total amount of protein per well, amount of expressed protein per total protein, Western blots, ELISA's, and mRNA expression. Preparation of the nucleic acid for transfection is critical in several applications, e.g., correct preparation of siRNA can be accomplished more easily by using a kit such as X-tremeGENE siRNA Dicer Kit. Examples of the above will be given for DOTAP, DOSPER, X-tremeGENE Q2, X-tremeGENE siRNA and/or FuGENE 6 Transfection Reagent. - X-tremeGENE is a trademark of a member of the Roche Group. - FuGENE is a trademark of Fugent, L.L.C., USA.

W-14

Higher Content Fluorescent Assays. JURGEN F. M. VANHAUWE. Invitrogen Corporation, Chicago, IL 60640. Email: jurgen.vanhauwe@invitrogen.com

Fluorescence has been around for quite some time now and multiple applications and detection methods have been devised to answer questions around structure and function of proteins as well as cell function. This workshop session will review some strategies to gain more information from fluorescent-based applications and its utility for biomarker discovery. Multiplexing of fluorescent assays has enabled researchers to analyze multiple samples or gain more confidence in the data and their resulting conclusions. Specific examples will be reviewed that address how multiple proteins can be analyzed at once and how kinetic measurements can be combined with end-point determinations. Additional data will be provided for using fluorescent polarization and time-resolved fluorometry in biomarker analysis. Lastly, analysis of serum samples for biomarkers discovery using fluorescent detection will be addressed for specific disease models.

W-15

Fluorescent Proteins for Transgenic Plants. C. NEAL STEWART, JR. Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996. Email: nealstewart@utk.edu

The cloning and subsequent expression of *Aequoria victoria* GFP (AvGFP) in transgenic organisms set off the fluorescent protein revolution. Up until about four years ago, the color palette and donor organism was largely limited to green, and a jellyfish, respectfully. Still, AvGFP had immense power in enabling the labeling of organelles, cells, organs, or even whole intact organisms. Recent years have brought about an explosion in the number of FP colors and organisms of origin. While there is much diversity in peptide sequences, the beta can structure is fairly conserved, but is variable in size and composition. Just as AvGFP was subject to repeated mutations to shift excitation and emission spectra and other characteristics, so have newer FPs. Thus, the pragmatic problem for the plant biologist has switched in recent years from a simple monochromatic limitation of AvGFP, to a glut of colors, variants, and sources; monomers, dimers, and tetramers; and corporate and university sources. Multi-labeling, FRET, and other choices are now available in many forms and combinations, but which FPs are most appropriate? The workshop session will review the FPs from corals and those named from fruits (e.g., tdTomato and mOrange), among others, as well as terminology, so that the end user can appropriately choose a new FP for the intended purpose at hand. In addition, tools for macroscopic detection will be described.

W-16

Chloroplast Genomics and Genetic Engineering. HENRY DANIELL. University of Central Florida, Department of Molecular Biology & Microbiology, Biomolecular Science Building 20, Room 336, Orlando, FL 32816-2364. Email: daniell@mail.ucf.edu

Chloroplast genetic engineering approach offers a number of unique advantages, including high-level transgene expression, multi-gene engineering in a single transformation event, transgene containment via maternal inheritance, ability to efficiently remove selectable marker genes, lack of gene silencing, position & pleiotropic effects and undesirable foreign DNA. More than forty transgenes have been stably integrated and expressed via the tobacco plastome to confer desired agronomic traits or express high levels of vaccine antigens and biopharmaceuticals. Despite such significant progress, this technology has not been extended to major crops. However, highly efficient carrot and cotton plastid transformation has been recently accomplished via somatic embryogenesis using species-specific chloroplast vectors, after bombardment of cultured cells containing non-green plastids. Chloroplast transgenic carrot plants withstand salt concentrations that only halophytes could tolerate. Future success will depend on the availability of the plastid genome sequences and the ability to regenerate homoplasmic transgenic lines in recalcitrant crops. Unfortunately, among 200 chloroplast genomes that are currently sequenced, only six are from crop species. Therefore, the complete chloroplast genome sequence of soybean, tomato, potato and genome evolution among legumes or Solanaceous crops will be discussed. Similarly, complete chloroplast genome of barley and sorghum will be presented and genome evolution among cereal crops will be discussed. In addition to summarizing progress in genomics, this presentation will focus on engineering cytoplasmic male sterility via the chloroplast genome. Engineered CMS phenotype was successfully reversed by continuous illumination. This approach offers a new tool for transgene containment, in addition to maternal inheritance and provides an expedient mechanism to produce large populations of male-sterile plants for F1 hybrid seed production.

W-17

Efficacy and Functionality of Chloroplast-derived Anthrax Protective Antigen. VIJAY KOYA¹, Stephen H. Leppla², and Henry Daniell. ¹Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science Building 20, Room 336 Orlando, FL 32816-2364 and ²Microbial Pathogenesis Section, National Institute of Allergy and Infectious Diseases, NIH, Building 30, Room 303, 30 Convent Dr., Bethesda, MD 20892-4350. Email: daniell@mail.ucf.edu

Anthrax, a fatal bacterial infection, is caused by *Bacillus anthracis*. The CDC lists anthrax as a Category A biological agent. The currently available human vaccine is prepared from alum-adsorbed, formalin-treated culture supernatant of a toxigenic, non-encapsulated strain of *Bacillus anthracis*. In addition to the key protective antigen (PA83) component, there are traces of the lethal and edema factors, and these may contribute to adverse side effects. In an effort to produce anthrax vaccine in large quantities and free of contaminants, PA83 was expressed in transgenic chloroplasts of *Nicotiana tabacum* var. petit Havana, by inserting the *pag* (2205 bp) gene into the chloroplast genome by homologous recombination. The pLD-VK1 vector contains *pag* with an N-terminal his-tag cloned into a universal chloroplast vector, pLD-ctv, that contains the 16S rRNA promoter, *aadA* gene coding for the spectinomycin selectable marker, *psbA* 5' & 3' untranslated regions to enhance translation in the light, and *trnI*, *trnA* homologous flanking sequences for site specific integration into the chloroplast genome. Chloroplast integration of the *pag* gene was confirmed by PCR and Southern analysis. PA83 protein expression was confirmed by immunoblot analysis and quantified by ELISA. Mature leaves contained up to 14.17% PA83 in total soluble protein when grown under continuous illumination. The PA83 was purified by affinity chromatography. The *in vitro* macrophage lysis assays showed that the crude extracts contained up to 20 micrograms/ml of functional PA83. Subcutaneous immunization with purified chloroplast-derived PA83 yielded IgG titers up to 1:320,000, similar to that of the group immunized with PA83 derived from *Bacillus anthracis*. Toxin neutralization studies and toxin challenge of immunized animals are in progress. The high expression levels of PA83 and strong immune response may facilitate development of a more highly purified and therefore safer anthrax vaccine at a lower cost of production.

W-18

Plastid Transformation in the Monocotyledonous Cereal Crop, Rice (*Oryza sativa*). MINKYUN KIM, Sa Mi Lee, Hyunsup Chung, Kyungso Kang, Soon Hee Yoo, Xiang Ming Xu, Seung-Bum Lee, Jong-Joo Cheong, and Henry Daniell. ¹School of Agricultural Biotechnology, Seoul National University, San 56-1, Sillim-dong, Kwanak-gu, Seoul 151-792, KOREA and ²Department of Molecular Biology and Microbiology, University of Central Florida, Orlando, FL 32816-2364. Email: mkkim3@plaza.snu.ac.kr

Rice is the stable food crop for more than one third of global population. Extension of plastid transformation technology to cereal crops, including rice, offers a great promise for improved agronomic traits and production of pharmaceutical or nutritional value added food without gene containment problem. Yet, not a single fertile transplastomic plant has been achieved in any monocotyledonous plant species. We report here promising progress towards stable plastid transformation in rice. Plastid transformation of rice was accomplished through the biolistic bombardment of 3 week-old total calli obtained from the mature seeds of japonica cultivar, Hwa-Chung. The produced transplastomic rice plants showed stable integration and expression of the *aadA* and *sgfp* transgenes in plastids. Moreover, the transplastomic rice plants produced viable seeds, which transmit the transgenes to T1 progeny plants. However, a 100% transmission of the transgenes was not achieved, probably due to the heteroplasmic state of the T0 plastid transformants. Also, compared to dicotyledonous plant species, rice plastid transformation is quite inefficient. Potential limiting factors in the routine application of plastid transformation to rice include the use of undeveloped plastid (proplastid) as the transformation target and the lack of highly efficient subculture systems with sustained plant regeneration capability. Strategies to overcome the aforementioned problems will be presented.

W-19

Industrial Applications of Plastid Transformation: Purification & Processing Strategies. S. Leelavathi¹, A. Bharadwaj¹, A. Ghosh² and V. S. REDDY¹. ¹International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi—110 067, India and ²Institute of Microbial Technology, Sector 39-A, Chandigarh—160 036, INDIA. Email: vsreddy@icgeb.res.in

Transgenic plants offer several advantages over conventional methods for large-scale production of recombinant proteins useful in human and animal health. Plants can express, process and fold recombinant proteins in a biologically active form. High-level expression and efficient recovery of recombinant proteins are two major factors that determine the success of this approach. Although it is difficult to compare directly the expression levels of various biopharmaceuticals expressed in the nuclear vs. chloroplast engineered plants, the expression levels of foreign genes are generally high in transplastomic plants due to possible amplification of the foreign gene copy number and the existence of efficient transcription and translation machinery within the chloroplasts. We have transformed two genes: 1) a thermostable xylanase, useful in paper, animal feed and bakery industry and 2) a human interferon gamma, pharmaceutically important therapeutic molecule into tobacco chloroplast genome. We have tried a number of strategies to over express these recombinant proteins and recover them from relatively large amounts of plant biomass. Our results indicate that the chloroplast genetic engineering coupled with expression strategies that reduce number of steps in downstream process hold the key for successful application of this approach. Results on the expression of recombinant molecules as fusion proteins and their advantage in the downstream process will be presented. Also use of thermostable property of xylanase in the over expression and purification of recombinant enzymes that are thermolabile will be presented.

W-20

Status of NICOSAN[™]/HEMOXIN[™], an Extract of a Mixture of Plants, for the Treatment of Sickle Cell Disease (SCD). R. C. PANDEY¹, P. Tripathi¹, R. Misra¹, P. N. Gillette², and T. Asakura³. ¹Xechem, Inc., New Brunswick, NJ 08901; ²State University of NY, Brooklyn, NY 11203; and ³Children's Hospital of Philadelphia, Philadelphia, PA 19104. Email: Ramesh@Xechem.com

NICOSAN[™]/HEMOXIN[™] (formerly NIPRISAN) is an extract of a botanical herbal mixture for treatment of sickle cell disease (SCD). Traditional health practices flourish in Africa, India (Ayurveda/Siddha) and China. Rev. P. O. Ogunyale in Nigeria had developed a herbal recipe of four herbs that appeared to benefit his patients who suffered from SCD. Studies have been done since 1998 at NIPRD, Abuja, Nigeria and the NIH Hemoglobin Reference Laboratory in Philadelphia, PA. Preclinical animal studies demonstrated no short term toxicity on gross behavior, response to pain, inflammation, liver, kidney, isolated rat atrium, frog rectus abdominis, guinea pig ileum and rabbit jejunum. Profound anti-sickling activity was identified which reduced the polymerization of deoxy-hemoglobin S *in vitro*, showed potent antisickling effects on sickle erythrocytes obtained from patients with SCD, and prolonged survival of transgenic sickle cell mice under hypoxic conditions under which all untreated mice died. A small, double-blind, placebo-controlled, randomized, crossover clinical trial of NIPRISAN in patients with SCD in Nigeria showed decreased frequency of painful crises. Serious toxicity was not found although rash, gastroesophageal reflux and diarrhea were described, and one patient had possible hemolysis. Standardization is in progress. Xechem has planned a full-scale validation and reliability program for both preclinical and clinical studies.

W-21

Discovery of the Cancer Chemopreventive and Hypocholesterolemic Properties of Pterostilbene A. M. RIMANDO. U.S. Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit, P.O. Box 8048, University, MS 38677. Email: arimando@msa-oxford.ars.usda.gov

Pterostilbene is a dimethylether analogue of resveratrol. Resveratrol has attracted much research interest due to its reported cancer chemopreventive and antioxidant properties. Its presence in grapes and wine has been associated with low incidence of death from coronary heart disease in populations who consume wine in moderate levels. The antioxidant and cancer chemopreventive activities of pterostilbene were investigated, triggered by its structural similarity with resveratrol, and was found to be similar to those of resveratrol. The inhibitory effect of resveratrol and pterostilbene on cytochrome P450 (CYP) dependent enzyme 7-ethoxyresorufin *O*-deethylase (EROD) was also investigated to demonstrate a possible mechanism of cancer chemopreventive activity. Pterostilbene was a potent inhibitor of CYP1A2. It inhibited EROD activity ($K_i = 0.39 \mu\text{M}$) in a mixed (competitive/noncompetitive) manner while resveratrol was only a weak inhibitor of this enzymatic marker ($K_i = 5.33 \mu\text{M}$). The reported hypocholesterolemic property of resveratrol generated interest to study its activation of the alpha isoform of the transcription factor peroxisome proliferator-activated receptor (PPAR), which is predominantly involved in fatty acid and lipid catabolism and import. Again, owing to structural similarity, the activation of PPAR alpha by pterostilbene was also investigated. *In vitro* studies showed that pterostilbene, but not resveratrol, activated endogenous PPARa in H4IIEC3 (rat hepatoma cell line) cells. *In vivo* studies showed 29% lower plasma low density lipoprotein cholesterol, 7% higher plasma high density lipoprotein cholesterol, and 14% lower plasma glucose in pterostilbene-fed hamsters given high cholesterol diet, compared to control group. Results obtained over a number of years of research provided evidence on health beneficial properties of pterostilbene, although it was not the HIV-1 reverse transcriptase inhibitory compound from the plant it was originally isolated from and studied for.

W-22

Studies on the Anti-tumor and Immuno-modulatory Mechanisms of *Scutellaria*-derived Phytochemicals in Tissue Culture and Animal Models. PRAHLAD PARAJULI¹, Nirmal Joshee², Agnes Rimando³, and Anand K. Yadav². ¹Neurological Surgery, Karmanos Cancer Institute & Wayne State University, Detroit, MI 48201; ²Agriculture Research Station, Fort Valley State University, Fort Valley, GA 31030; and ³USDA, Natural Products Utilization Research Unit, POB 8048, University, MS 38677. Email: pparajuli@med.wayne.edu

At least 7,000 medicinal compounds in the Western pharmacopoeia are derived from plant sources. Of these phytochemicals, about 75% show a positive correlation between their modern therapeutic uses and the traditional applications of the plant species they are derived from. New phytochemicals are being continuously introduced as dietary supplements. However, as many people who use these traditional medicines and dietary supplements do not consider them to be a part of their "medication," inadvertent side-reactions and even fatality might occur as some of the plant products react with other medications that the patient might be taking. It is, therefore, extremely important that new plant products be thoroughly investigated in order to characterize their active ingredients, mechanisms of action, and potential drug interactions. Our group is studying the anti-tumor and immuno-modulatory mechanisms of phytochemicals derived from *Scutellaria*. In this presentation, we will discuss some of the *in vitro* and *in vivo* studies for the characterization of biological properties of novel herbal compounds illustrating examples from our ongoing studies on *Scutellaria*.

P-1000

Large Scale *In Vitro* Bulblet Production in Endemic and Endangered Flower-bulbs *Sternbergia candida* and *Muscari muscarimi*. SEBAHATTIN ÖZCAN, İskender Parmaksız¹, Semra Mirici³, Sati Çöçü¹, Cengiz Sancak¹, Serkan Ur-anbey¹, Ercüment O. Sarihan¹, Bilal Gürbüz¹, Cafer S. Sevimay¹, & Neşet Arslan¹. ¹Department of Field Crops, Faculty of Agriculture, University of Ankara, 06110, Dışkapi, Ankara, TURKEY; ²Department of Biology, Faculty of Science and Arts, University of Gaziosmanpaşa, Tokat, TURKEY; and ³Faculty of Education, University of Akdeniz, Antalya, TURKEY. Email: ozcan@agri.ankara.edu.tr

Sternbergia candida and *Muscari muscarimi* are important ornamental plants because of their attractive yellow flowers which open in early spring and have pleasant smell. Besides use as ornamental plants, they have also a great potential in perfume and pharmaceutical industry. However, they are endemic and endangered species of Turkey and threatened by complete extinction. In recent years international agreements have been employed throughout the world for the protection of endangered geophytes and therefore collection of *S. candida* and *M. muscarimi* from the natural habitats is now forbidden in Turkey. The natural propagation rate of most geophytes including *S. candida* and *M. muscarimi* is relatively low. This often hampers the large-scale cultivation of these plants. We overcame this problem by tissue culture techniques. Bulb scale and immature embryo explants of *S. candida* and *M. muscarimi* were cultured on different nutrient media supplemented with various concentrations of plant growth regulators. Immature embryos produced higher number of bulblets than bulb scales. Large numbers of bulblets were produced (over 100 bulblets/explants) from immature embryos on MS medium supplemented with 4 mg l⁻¹ 6-benzylaminopurine (BAP) and 0.25 mg l⁻¹ α-naphthaleneacetic (NAA) or 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) after 12-14 months of culture initiation. Regenerated bulblets were kept at 5° C for 5 weeks and then transplanted to a potting mixture. To our knowledge the present study is the first report for *in vitro* bulblet production from immature embryos of geophytes. The procedure described here provides a prolific bulblet regeneration system that may form the basis of bioreactor culture of *S. candida* and *M. muscarimi*.

P-1001

Efficient Micropropagation Protocol of an Antimalarial Plant - *Spilanthes acmella* L. var *oleracea*. VIBHA PANDEY, Kuldeep Sharma, and Veena Agrawal. Department of Botany, University of Delhi, Delhi 110007, INDIA. Email: drveena_du@yahoo.co.in

Spilanthes acmella L. var *oleracea* Clarke (Asteraceae), commonly known as 'akarkara' is a valuable medicinal herb containing strong antimalarial, antibacterial and antifungal activity. Besides it is extensively used in treating toothaches, throat infection, gums and urinary tract diseases. The plant owes its activity to the bioactive compound spilanthol and a number of immune stimulating alkylamides. *In vitro* regeneration of this potential herb has been achieved through leaf explants excised from *in vitro* raised shoots, on MS medium augmented with a variety of growth regulators such as auxins (2,4-D, NAA & IBA) and cytokinins (BA & Kn) tried either alone or in combination. MS + 1 μM NAA + 10 μM BA proved optimum for differentiating an average of 12.90 ± 0.32 shoot buds per responding culture in 60% explants. An elevation in average shoot length (2.53 ± 0.45) along with further proliferation of shoot buds (15.7 ± 0.70) was observed when the shoots were subcultured on MS + 10 μM BA. Such buds on transfer to MS + 1 μM NAA + 1 μM BA showed significant enhancement in shoot length (5.27 ± 1.0) in 100% cultures. For rhizogenesis, MS (1/2) + 0.1 μM IBA proved optimum, where 100% shoots developed an average of 19 ± 0.56 roots within 15 days. The plants were successfully transferred to greenhouse after initial hardening. 100% of the plants showed normal flowering without any morphological variation. Thus, an efficient micropropagation protocol has been established from lab to land transfer of plants in this valuable taxon.

P-1002

Plant Regeneration in Guava Through Somatic Embryogenesis. B. BISWAS, N. Joshee, A. Yadav, and A. K. Yadav. Agricultural Research Station, Fort Valley State University, Fort Valley, GA 31030-4313. Email: biswasb@fvsu.edu

Guava (*Psidium guajava* L., Myrtaceae) is an exotic fruit with excellent nutraceutical values. This plant native to warm climates cannot be cultivated in temperate zone especially with severe winters. Hence, genetic improvement of guava particularly for cold tolerance is indispensable. In order to apply biotechnology through genetic transformation to enhance cold hardiness, a reliable and rapid guava micropropagation system is needed. With that, developing the most dependable micropropagation protocols for guava was the primary purpose of this research. Guava explants using different tissues like leaf, node, internode, anther, petal, mesocarp and immature zygotic embryos, from mature trees of 'Gushiken Sweet' guava growing in the specialty plants house were prepared. Since we did not get embryogenesis in the media like MS, B5, N6, etc., a modified medium, viz., guava somatic embryogenesis medium (GSEM) was developed. Of all explants investigated, only immature zygotic embryo produced a high frequency of SE on GSEM when supplemented with 58.4 μM L-glutamine and 1.75 μM IAA under 10 h photoperiod and ambient temperature. The resulting SE produced secondary SE upon further culturing under same conditions. However, if these SE were not routinely subcultured, they spontaneously germinated and developed normal healthy plantlets. Moreover, these SE germinated and developed normal healthy plantlets upon timely subculturing in less expensive MS medium. Synthetic seeds were developed using encapsulation of SE with sodium alginate. Besides, timed observations on these synthetic seeds for germination and development of plantlets are under way.

P-1003

Comparison of Methods of Liquid Medium Culture and Effects of Temporary Immersion of Explants for Banana (cv Dwarf Cavendish) Micropropagation. FARAH FARAHANI¹, Ahmad Majd², Reza Zarghani³. ¹Dept. of Biology, Fac. of Science Islamic Azad Univ. Qom unit, Qom; ²Dept of Biology, Tarbiat Moalm University, Tehran, IRAN; and ³Agriculture of Research Biotechnology Institute Karaj, IRAN. Email: farahfarahani2000@yahoo.com

Four different liquid medium culture methods for meristem propagation of bananas were investigated and compared with solid medium culture. Treatments studied were: solid culture medium (A), liquid medium with immersion of the plants (B), liquid medium with cotton culture support (C), liquid medium aerated by bubbling (D), liquid medium with temporary immersion of the explants for 20 min every 1 h (E). After 4 weeks of culture, shoots in liquid medium with immersion and liquid medium aerated by bubbling proliferated little or not at all, shoots on solid medium and those subjected in liquid medium with cotton culture support displayed multiplication rates of 2.7 to 3.5, and the highest multiplication rate (>7) was observed in explants subjected to temporary immersion in the medium. Three groups of treatments differed in the accumulation of dry matter: the smallest weight (around 0.6 g) was observed in treatment B and D, and accumulation was 2 to 4 times greater in the explants in solid culture medium and those subjected to liquid medium with cotton culture support. The highest multiplication rates and weight gains were observed in liquid medium with temporary immersion (E). Shoots in liquid medium continuously aerated by bubbling displayed hyperhydricity of the outer leaf sheaths. This was not observed with temporary immersion of explants.

P-1004

Direct Shoot Bud Differentiation and Plantlet Regeneration from Leaf and Petiole Explants of *Begonia tuberhybrida*. S. Nada¹, Siva Chennareddy¹, R. V. Sairam, S. Zhang, K. MEEKER, T. V. Reddy, and S. L. Goldman. Plant Science Research Center, University of Toledo, Toledo, OH 43606. Email: srudrab@utnet.utoledo

The genus *Begonia* contains around 2000 species and belongs to the family Begoniaceae. Begonias are one of the most popular ornamental plants in the world and are used as garden plants, potted plants, hanging baskets and greenhouse flowers. Begonia is one of the top bedding/potted plants in Ohio and USA. Information on Begonia tissue culture is limited, in general and particularly for *Begonia tuberhybrida*. We report here a novel high frequency protocol for *in vitro* plant regeneration from leaf and petiole segments of *Begonia tuberhybrida* through direct shoot bud formation without intervening callus phase that resulted in complete regeneration of plantlets within three months. The explants were incubated at 16/8-hour dark photoperiod provided by cool-white fluorescent lights at a quantum flux density of 30 $\mu\text{mol s}^{-1} \text{m}^{-2}$. Shoot buds were induced directly on the adaxial surface of leaf tissues from not only at the cut ends but also across the entire surface of both leaf and petiole segments. The highest frequency of shoot bud formation was 90%, and the maximum number of shoots (132) per leaf explant was achieved with modified Murashige and Skoog media supplemented with 1.0 mg/l NAA (α -naphthalene acetic acid) and 2.0 mg/l TDZ (Thidiazuron). In petioles, the highest frequency of shoot buds was 82%, and maximum number of shoots (33) per explant was achieved with 0.5 mg/l NAA and 2.0 mg/l TDZ. The number of shoots produced in both the explants was drastically reduced in the treatment with BAP alone or in combination with NAA and/or TDZ. The regenerated shoots were rooted on MS medium supplemented with 0.5 mg/l NAA. MS basal medium without hormones had no effect on shoot elongation, while MS basal medium supplemented with 0.5 mg/l GA₃ had good positive effect on shoot elongation. All the elongated shoots developed into complete rooted plantlets within three months. All rooted plantlets were successfully transferred to soil in pots in the greenhouse and they appeared morphologically normal and flowered in the greenhouse.

¹These authors contributed equally to the manuscript.

P-1005

Extensive Field Evaluation of Staple Food Crop Varieties on Atolls: Collected Around the Globe and Multiplied by Tissue Culture. VIRENDRA MOHAN VERMA¹, John J. Cho², Phillip Jackson³, Jabukja Aikne¹, and Jina David¹. ¹Co-operative Research and Extension (USDA Land Grant), College of the Marshall Islands, Majuro MH 96960, MARSHALL ISLANDS; ²Department of Plant Pathology, HITAHR-Maui County Research, University of Hawaii, Kula, Maui, HI 96790; and ³College of Micronesia-FSM, Pohnpei, FM 96941, MICRONESIA. Email: vmv_vmv@hotmail.com

The USDA approved projects were developed on tissue culture of staple food crops aiming towards introduction and field evaluation in the Marshall Islands to overcome the limitations of non-availability of elite and disease-free planting material and adequate soil management techniques. Micronesian atolls are low-lying coral atolls scattered in the central Pacific Ocean. The alkaline make-up of atoll soils results in less availability of plant nutrients, especially iron, manganese, copper, and zinc, which limits the growth of many introduced plants. In addition to these, soils are also deficient in nitrogen and/or phosphorus and all lack sufficient potassium. The germplasm of taro and sweetpotato that originally belongs to Thailand, Malaysia, Indonesia, Fiji, Papua New Guinea, Samoa, Hawaii, Philippines, Solomon Islands, Palau, Taiwan, Marshall Islands, Guam, New Caledonia, Vanuatu and Tahiti, was multiplied through tissue culture using the protocol developed and presented as contributed paper in 2004 World Congress on In Vitro Biology by the first author of this paper. Thousands of elite and disease-free plants were multiplied for extensive field evaluation and distribution to growers. The results of research are excellent and very encouraging for atoll growers. This success has resulted in expansion of the project throughout Micronesia by another WSARE-USDA approved project. This study indicates that the tissue culture is the only way to generate elite and disease-free plants as per demand and the potential of sweetpotato and taro cultivation on atolls is fair. However, specific techniques of soil fertility management through compost application in combination with a little amount of chemical fertilizer, cultivation strategies and proper irrigation are very critical, considering the nutrient deficient, porous nature of atoll soils.

P-1006

In Vitro Seed Germination and Seedling Development of *Habenaria macroceratitis*, an Endangered Terrestrial Orchid. S. L. STEWART and M. E. Kane. Environmental Horticulture Department, University of Florida, PO Box 110675, Gainesville, FL 32611. Email: sstewart@ifas.ufl.edu

Habenaria macroceratitis, a rare terrestrial orchid restricted to a few sites in central peninsular Florida, requires a stable, undisturbed habitat to flourish; however, habitat conversion to agricultural lands and home sites has caused populations to decline. A series of *in vitro* culture experiments was designed to: 1) characterize *in vitro* seed germination, 2) study the effects of cytokinins on seed germination, and 3) explore the effects of photoperiod on *in vitro* seedling development. In a screen of asymbiotic germination media, Malmgren's Modified Terrestrial Orchid Medium (MM) supported the greatest germination percentage (80.9%), whereas Knudson's C (78.5%), Vacin and Went (67.8%), and 1/2-strength Murashige and Skoog (55.3%) supported lower percent germination. Contrary to the dogma that terrestrial orchid seeds require dark incubation to stimulate germination, no significant effect of photoperiod (24/0 h L/D, 16/8 h L/D, 0/24 h L/D) was found in any treatment condition. Little variation in asymbiotic seed germination percentage was observed on MM supplemented with BA, kinetin, zeatin, or 2-iP at 0, 1, 3, or 10 μM . However, a significant variation in protocorm morphology was observed. Photoperiodic effects (16/8 h L/D, 12/12 h L/D, 8/16 h L/D) on *in vitro* seedling development were tested. Significant increases in leaf length, leaf width, tuber diameter, plant fresh weight, tuber fresh weight, and tuber dry weight, and a significant decrease in leaf number were observed in 8/16 h versus 16/8 h photoperiod. The 12/12 h photoperiod was intermediate in all measured variables. An integrated conservation plan is being developed using data generated in these experiments, as well as information from ecological and molecular studies.

P-1007

In Vitro Germination and Seedling Development of *Calopogon tuberosus*. P. J. KAUTH, W. A. Vendrame, and M. E. Kane. Environmental Horticulture Department, University of Florida, PO Box 110675, Gainesville, FL 32611. Email: pkauth@ufl.edu

Many native terrestrial orchids exhibit commercially valuable characteristics but, unlike epiphytic species, seed culture protocols have not been well defined for commercial production. The influence of culture media and photoperiod (16 h L/8 h D and 24-h D) on *in vitro* seed germination and seedling development in *Calopogon tuberosus*, a common terrestrial orchid of eastern North America, was evaluated. Germination percentages of surface sterilized seed and subsequent seedling growth and development were compared weekly for 8 weeks on Knudson C (KC), Malmgren's Modified Terrestrial Orchid Medium (MM), and PhytoTech Mother Flasking II Medium (P723). Seedling development was assessed based on the six developmental stages described by Stenberg and Kane (1998). Germination percentages on the three media were similar between seed cultured in light and dark. After 8 weeks, total seed germination percentages were as follows: KC light (41.8%) and dark (45.2%), MM light (31.6%) and dark (39.9%), and P723 light (27.7%) and dark (25.4%). However, seedling development was significantly affected by culture media and light conditions. After 8 weeks only seedlings cultured on either P723 (19.8%) or KC (0.2%) in light developed to Stage 6 (seedlings with ≤ 3 leaves with roots evident). Seedlings transferred to fresh media after 16 weeks in culture will be assayed for subsequent growth after 24 weeks. Preliminary results show that *C. tuberosus* grows rapidly from mature seed, however seedlings inoculated on P723 and incubated developed more rapidly than those on KC and MM. Since *C. tuberosus* grows rapidly in culture, the orchid is being used as a model organism for future *in vitro* experiments.

P-1008

Efficient Organogenesis and Somatic Embryogenesis and Mean Metabolic Heat and RCO_2 Production Rates Measured by Microcalorimetry During Organogenic and Somatic Embryogenic Induction Processes in *Cineraria* 'Jester Pink.' David W. Burger², Heiner Lieth², GYEONG HEE KIM¹, and Byoung Ryong Jeong¹. ¹Dept. of Horticulture, Division of Applied Life Science, Graduate School, Gyeongsang National University, Jinju 660-701, KOREA and ²Dept. of Environmental Horticulture, University of California, One Shields Avenue, Davis, CA 95616. Email: nany96@hanmail.net; brjeong@gsnu.ac.kr

Cineraria is a species belonging to the family Asteraceae and is a colorful pot crop. Somatic embryogenesis in *Cineraria* can be used as a tool for clonal propagation eliminating the problem of propagule segregation. In *Cineraria*, only a few reports on adventitious shoots induction and somatic embryogenesis have been published, and somatic embryo production via embryogenic calli induction, suspension culture, and transformation are yet to be achieved. In order to optimize the culture conditions for mass propagation of *Cineraria* through somatic embryogenesis, the effect of NAA and 2,4-D on the induction of friable embryogenic calli and somatic embryos from different explants was investigated. 13.2 μM NAA alone induced rhizogenesis, while 13.2 μM NAA + 4.5 μM BAP induced nodular structures, without an intervening callus phase, which then developed into shoots. 13.2 μM 2,4-D alone or in combination with 4.5 μM BAP induced somatic embryos directly from the surface of leaf explants without an intervening callus phase. Explants on media containing BAP only swelled or turned brown. We also examined heat production and CO_2 production rates by cultured cotyledons for 5 weeks of different developmental processes, such as somatic embryogenesis and organogenesis, by using a microcalorimeter. Mean heat production and mean CO_2 production rates by all tissues increased after 1 week of treatment. Mean heat production and mean CO_2 production rates by somatic embryogenic (13.2 μM 2,4-D + 4.5 μM BAP or 13.2 μM 2,4-D) and by organogenic (13.2 μM NAA + 4.5 μM BAP or 13.2 μM NAA) treatments kept increasing or remained high thereafter, while they decreased or remained constant in the other treatments such as PGR-free treatment. The presence of the PGR had effect on metabolic activity and morphogenesis in *Cineraria*.

P-1009

Growth and Sugar Use in Agar and Liquid with Large and Small Vessels Using Turmeric (*Curcuma longa*) as a Model Crop. JEFFREY ADELBERG. Department of Horticulture, Clemson University, Clemson, SC 29634. Email: jadlbrg@clemson.edu

Comparisons of agar and liquid tissue culture systems are often confounded by dissimilar methods (e.g. vessel size, plant spacing, agitation, explant/media volume ratio). In this study, turmeric was grown at a density of 6-ml per explant allowing direct comparison among treatments. Agar (7 g/l) and liquid media were dispensed in large (5.5-l) Nalgene Biosafe and small jars (180 ml) as vessels. Liquid medium was maintained in agitated and stationary phases. Plants on agar were placed in uniform and non-uniform arrays (similar to spacing to liquid). On agar, fresh weight increased 7 \times and dry weight was 75 mg per explant, regardless of treatment. Non-uniform placement of explants caused non-equilibrium distribution of sugar that was greater in large vessels. Plant quality (% dry) was directly related to sugar concentration at medium surface adjacent to plant ($r^2=0.51$). Sugar use did not correlate to dry, or water weight of plants on agar. In stationary and agitated liquid medium, there was a 5.3 \times and 12 \times increase, respectively, in fresh weight. In smaller vessels of liquid, agitation increased dry weight from 34 to 97 mg per explant. In larger vessels of liquid, agitation increased dry weight from 116 to 140 mg per explant. Large vessels and agitation increased sugar use and there was a direct relation between sugar use and dry weight ($r^2 = 0.96$) and sugar use and water weight ($r^2=0.96$) of plants in liquid. Sugar was uniformly distributed in liquid medium and plants were 7.5% dry weight. Agitated liquid medium in small vessels had 2.9 \times increase in numbers of plants; the other liquid medium treatments had 2 \times , and agar medium had 1.2 \times increase. Agitating liquid medium increased fresh and dry weight compared to stationary liquid, or agar. Larger vessels of agitated liquid had largest increases in plant mass, small vessels of agitated liquid had greatest increases in numbers of plants. Despite a non-uniform placement of explants, agitated liquid medium allows greater sugar and water use than manual uniform distribution of plants on agar.

P-1010

Abstract has been withdrawn.

P-1011

Movement and Containment of Microbial Contamination in the Nutrient Mist Bioreactor. M. A. SHARAF-ELDIN and P. J. Weathers. Biology and Biotechnology Dept., Worcester Polytechnic Institute, Worcester, MA 01609. Email: weathers@wpi.edu

Since they are cultured in such a sugar-rich medium, often for more than 3 weeks, *in vitro* plant cultures are easily contaminated by fungi and bacteria. Contamination of bioreactors is especially problematic as larger volumes entail larger losses. Common microbial contaminants are the spore forming *Penicillium* fungi and *Bacillus* bacteria. To study the movement and subsequent control of contaminants in the mist bioreactor, *Penicillium chrysogenum*, or *Bacillus subtilis* were deliberately inoculated into three possible locations in the reactor: the Growth Chamber (GC), the medium Reservoir (R), or the mist generating chamber (Mist Generator; MG). Compared to deliberate inoculation of *P. chrysogenum* into either the R or MG region, fungal growth originating in the GC required three more days (~ 60% more time) to move throughout the rest of the reactor. In contrast, regardless of where *B. subtilis* was inoculated (GC, R, or MG), it took 7 days to contaminate the entire system. The movement of filamentous microbes that are not sporulating is, thus, quite different from bacteria. Although neither contaminant, once visibly present in the reactor, was controllable by subsequent addition of Plant Preservatives Mixture (PPM), both microbes are completely inhibited if PPM is added to the sum of the MG at the time of inoculation in the GC. Experiments are now in progress using PPM to determine if microbial control is possible without also damaging plant cultures using a species sensitive to PPM (*Arabidopsis*), or insensitive to PPM (*Artemisia*). These results will prove useful in the implementation of large-scale *in vitro* culture systems.

P-1012

Physical Interactions Between Aminoglycoside Antibiotics and Gelrite: Documentation and Effects on Antibiotic Efficacy. M. J. BOSELA. Department of Biology, Indiana University-Purdue University at Fort Wayne, 2101 E. Coliseum Boulevard, Fort Wayne, IN 46805-1499. Email: boselam@ipfw.edu

Antibiotics from the aminoglycoside family, such as kanamycin, hygromycin, and Geneticin G-418, are routinely used as selective agents for plant transformation. In general, hygromycin and G-418 confer highly stringent selection. However, the efficacy of kanamycin, which is less directly toxic to plant tissues, is more variable, and has been reported to be affected by the type of gelling agent employed, with higher kanamycin concentrations being required when Gelrite or carrageenan are used in place of agar (Wilmink and Dons, 1993, *Plant Molecular Biology Reporter* 11:165-185; Chauvin et al., 1999, *Plant Cell Tissue and Organ Culture* 58:213-217). The lower efficacy of these alternative media types has been variously attributed to antibiotic precipitation (Kapaun and Cheng, 1999, *HortScience* 34:727-729) and/or antibiotic 'binding' by the gelling agent(s) (Wilmink and Dons, 1993; Charvin et al., 1999). The current research was initiated to clarify the types of interactions possible between aminoglycoside antibiotics and Gelrite, and to quantify their effects on antibiotic efficacy. Precipitation, appearing as suspensions of colloidal crystals, has been observed for both media types (agar and Gelrite). In contrast, antibiotic binding has only been observed for Gelrite media, with the binding taking the form of Gelrite crosslinking by the antibiotic resulting in the production of bubbles or tubes of precocious (flash) gelling, in severe cases, and/or heightened rates of gel setting. Both types of interaction are specific to aminoglycoside antibiotics, with the degree of reaction (precipitation or binding/gelling) being related to the nutrient salt concentration of the media, the number of amino groups on the antibiotic, and the antibiotic charge. The biological significance of these phenomena is being evaluated via the use of pH-adjusted antibiotic stocks to control antibiotic charge, and, in turn, the degree of precipitation or precocious gelling (Kapaun and Cheng, 1999).

P-1013

Optimum Ventilation in Culture Vessels Reduces the Hyperhydricity in Shoot Cultures of *Scrophularia yoshimurae*. HSIN-SHENG TSAY¹, Satisch Manohar Nalawade¹, Wei Fang², and Chien-Chou Lai². ¹Institute of Biotechnology, Chaoyang University of Technology, Wufong, Taichung 413 TAIWAN and ²Department of Bio-Industrial Mechatronics Engineering, National Taiwan University, Taipei 106, TAIWAN. Email: htsay@mail.cyut.edu.tw

An efficient method has been developed for obtaining healthy shoots via shoot organogenesis in *Scrophularia yoshimurae* using the nodal segments. Nodal segments cultured on Murashige and Skoog's (MS) basal medium supplemented with 1.0 mg/L benzyladenine (BA), 0.2 mg/L α -naphthaleneacetic acid (NAA) induced multiple shoots in different types of culture vessel closure systems tested. Higher numbers of shoots were hyperhydric/vitrified in the hermitically sealed culture vessels. The hyperhydricity of the shoot cultures was decreased by progressively ventilating the vessels. Higher amounts of ethylene and CO₂ accumulated in the miniature microenvironment (headspace) of hermitically sealed culture vessel containing cultures. A strong correlation between the number of adventitious shoot buds developed with the accumulation of ethylene and CO₂ in culture vessel has been observed. Hindered gaseous exchange affected the shoot growth both quantitatively and qualitatively. The exchange of gases could be achieved by removing the Parafilm sealing without causing any damage to the sterility. The high gaseous exchange rates increased the evapotranspiration and caused dehydration of proliferating nodal segments and culture medium. This also resulted in reduced hyperhydricity and influenced morphogenetic response. Our results demonstrate that healthy shoots *in vitro* proliferated shoot cultures of *Scrophularia yoshimurae* could be obtained by optimum gaseous exchange during culture.

P-1014

The Plasmid (pToK47) Containing *virB* and *virG* from *Agrobacterium tumefaciens* A281 Provide High Transformation Efficiency of *Hevea brasiliensis* Via *Agrobacterium*-mediation. P. AROKIARAJ, A. R. Shamsul Bahri, and R. Leelawathy. Malaysian Rubber Board, Rubber Research Institute of Malaysia Experiment Station, Biotechnology and Strategic Research Unit, 47000 Sungei Buloh, Selangor, MALAYSIA. Email: parokiaraj@lgm.gov.my

The ability of the supervirulence plasmid (pTok47) to enhance genetic transformation success in *Hevea* was investigated. The effect of the plasmid on the virulence of two strains of *Agrobacterium* (GV2260 and GV3850) harbouring the human serum albumin cDNA was tested on *Hevea* anther calli. Equal numbers of cultures ranging from 74 to 210 were initiated each day for each treatment. In the data analysis, the day to day effects were removed by pairing the (+) and (-) supervirulence treatments. As Gaussian distribution of the data was suspect, a non-parametric paired test, the Wilcoxon Signed Rank Test, was used in statistical evaluation for the effectiveness of the supervirulence plasmid in *Hevea* genetic transformation. The results of this study showed that both *Agrobacterium* strains benefited from the presence of the supervirulence plasmid in giving a higher frequency of transformed calli. These differences were significant at $p=0.0001$ for GV2260 and $p=0.01$ for GV3850. GV2260 with supervirulence also gave a higher frequency of embryoid formation significant at $p=0.003$, although a similar advantage was not reflected in GV3850 with the difference not being significant ($p=0.25$). These data suggest that a highly virulent binary vector system might prove especially useful in generating high frequency transformation of *Hevea*.

P-1015

Genetic Transformation and Transgenic Plant Recovery from Species of Grape. S. A. DHEKNEY, Z. T. Li, M. Dutt, M. Van Aman, and D. J. Gray. University of Florida/IFAS, Mid-Florida Research and Education Center, 2725 Binion Road, Apopka, FL 32703-8504. Email: sadanand@ifas.ufl.edu

Gene delivery into embryogenic cells using *Agrobacterium* is the most commonly used transformation method for grape. Although transformation of certain grape varieties has become routine in the last decade, hundreds of genotypes comprising several species have either been found to be recalcitrant or have not yet been tested. Factors that influenced *Agrobacterium*-mediated genetic transformation were studied in 15 *Vitis* scion varieties and three rootstocks. The Green Fluorescent Protein (GFP) reporter gene was utilized to facilitate optimization of experimental procedures. Different developmental stages of embryogenic cell cultures were evaluated for use as target cells for recovery of stable transformants. The effect of various antioxidants on transformation efficiency also was studied. Transient GFP expression levels ranged from 1% to 81% among species and varieties. A similar pattern was observed for stable expression. Transgenic plants have been recovered from *Vitis riparia* 'Riparia Gloire', *Vitis rotundifolia* 'Alachua', *Vitis rupestris* 'St. George', *Vitis vinifera* 'Cabernet Franc', 'Char-donnay', 'Merlot', 'Pinot Noir', 'Sauvignon Blanc', 'Superior Seedless', 'Thompson Seedless' and 'Zinfandel', *Vitis* hybrids 'Conquistador', 'Freedom' and 'Seyval Blanc'. For *V. vinifera* genotypes and *Vitis* hybrids, cotyledonary stage somatic embryos were found to be the best target material for co-cultivation with *Agrobacterium*; whereas, proembryonal masses and somatic embryos were best for 'Alachua', 'St. George' and 'Freedom'. No differences in transient expression were observed among different treatments when DTT (dithiothreitol) was used as an antioxidant, however the number of stably transformed calli and embryo lines was improved. Evaluations are underway for *V. vinifera* 'Autumn Seedless', 'Cabernet Sauvignon', 'Orange Muscat' and 'Shiraz'.

P-1016

Generation of Marker-free Transgenic Tobacco Plants Without the Application of Selection Pressure. BAOCHUN LI, Hui Qiu, and Huan Xie. Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY 40546-0236. Email: bli2@uky.edu

The presence of marker genes in transgenic plants for the production of plant-made pharmaceutical (PMP) or other agricultural application is undesirable. Tobacco (*Nicotiana tabacum* L.) and other *Nicotiana* species have shown great potential for PMP productions, and development of a marker-free transformation system for tobacco would be very beneficial for this application. We are interested in developing a marker-free tobacco transformation system by not applying selection pressure. In our study, leaf explants were infected with *Agrobacterium* carrying either a marker-less binary vector, or a binary vector with a marker gene. The infected explants were incubated on shoot-induction medium without selective compounds for shoot induction. The induced shoots were analyzed by PCR or *gusA* histochemical assay and those that were PCR and *gusA* positive were rooted and transferred to the greenhouse for T1 seed production. The T1 plants were assayed histochemically for *gusA* expression and inheritance, and by Southern hybridization to characterize the integration of transgenes. We have conducted six experiments, each involving three binary vectors and 50-100 leaf explants. T1 plants from 44 primary events have been assayed histochemically so far for *gusA* expression. T1 plants from 31 of the 44 events were found to be *gusA* positive. Of the 31 events, 26 transmitted the *gusA* activity to the T1 generation in a Mendelian 3:1 ratio. The T1 plants from more primary events are being assayed histochemically for *gusA* expression, and Southern analysis will be followed for those *gusA* positive events. The efficiencies of transformation and the percentage of escapes will also be determined.

P-1017

Factors Affecting *Agrobacterium*-mediated Sunflower Transformation. NIU DONG¹, Noemsha Williams¹, Colleen McMahan¹, Donna Rath², Calvin Pearson², and Katrina Cornish³. ¹USDA/ARS, Western Regional Research Center, Crop Improvement and Utilization, 800 Buchanan St., Albany, CA 94710; ²Colorado State University-Western Colorado Research Center-Fruita, Fruita, CO 81521; and ³Yulex Corporation, 1945 Camino Vida Roble, Suite C, Carlsbad, CA 92008. Email: ndong@pw.usda.gov

Sunflower is a recalcitrant plant species for transformation. Mature seeds from 7 genotypes were tested and #665 had the highest regeneration efficiency. To increase the transformation efficiency, a vector containing *bar* gene and *gus* gene with an intron was used. Different explants such as half apexes, or proximal parts of the cotyledons were tested. Wounding by different methods such as bombardment, shaking with grass beads, or sonication were also tested. Effect of cysteine was examined. Shoots developed from explants were selected by herbicide ammonia-glufosinate. The best result was obtained when using proximal parts of the cotyledons, sonicated for 2 × 15 seconds, and inclusion of 400 mg/L cysteine in both inoculation solution and co-culture medium. Three independent GUS positive shoots have been obtained.

P-1018

The Retention of an Engineered Virus in Plant Callus by Selection. V. GABA, S. Singer, and A. Gal-On. Dept. of Virology, Agricultural Research Organization Volcani Center, POB 6 Bet Dagan, 50250 ISRAEL. Email: vpgaba@volcani.agri.gov.il

In vitro-grown seedlings of melon (*Cucumis melo* L.) cv. Revival were inoculated with an engineered symptomless viral vector strain of Zucchini Yellow Mosaic Virus (AG), bearing the *bar* gene conferring field resistance in cucurbit crops to the herbicide Basta (Shiboleth et al., J. Biotechnol. 92, 37, 2001). Melon plants inoculated with the AG-bar vector grew *in vitro* on concentrations of phosphinothricin (PPT), the active ingredient of Basta, lethal to control (uninoculated) melon plants. Leaf explants from plants infected with AG-bar vector produced callus on a callus-forming medium with PPT of a concentration that prevented the formation and growth of callus from control plants. Callus bearing the AG-bar vector grew on medium containing a high concentration of PPT for 20 monthly cycles. Callus infected with the AG-bar virus generally grew more slowly on medium with PPT than on medium without the herbicide. Callus viral titer was measured by ELISA to detect the viral coat protein. Virions were visible by electron microscope in preparations from callus after several cycles in culture. After many transfers in culture, callus extracts were able to inoculate squash plants with the AG-bar viral strain. The level of viral protein in callus was compared to that found in infected melon leaves. When callus infected with the AG-bar vector was transferred to medium without PPT, the virus titer declined, and by the fifth passage without PPT the AG-bar vector was no longer detectable in the culture. This demonstrated that selection could enable the maintenance of a viral strain in culture that would otherwise be lost. After 20 transfers in culture, a few alterations were found in the *bar* gene of the AG-bar viral vector, without altering the protein sequence.

P-1019

Improvement in Transformation of Anthurium. M. FITCH¹, T. Leong², H. Albert¹, H. McCafferty², J. Zhu², K. Nickolov², T. Mangwende², P. Moore¹, and D. Gonsalves³. ¹Pacific Basin Agricultural Research Center, ARS, USDA, 99-193 Aiea Hts. Dr., Aiea, HI 96701; ²Hawaii Agriculture Research Center, 99-193 Aiea Hts. Dr., Aiea, HI 96701; and ³Pacific Basin Agricultural Research Center, ARS, USDA, 99 Aupuni St., Hilo, HI 96720. Email: MFITCH@PBARC.ARS.USDA.GOV

Although genetic transformation of Anthurium has been reported, the efficiency was relatively low and slow, requiring at least 12 months from the time of co-cultivation with *Agrobacterium tumefaciens* to regenerated transgenic plants (Chen and Kuehnle, 1996). We modified those published methods in an attempt to decrease the time and increase the efficiency for producing transgenic Anthurium plants. Embryogenic calli, initiated from leaf, stem, petiole, and root explants of *in vitro*-grown anthurium plants, were bright yellow in color and in the absence of selective pressure, capable of regeneration into green plants in about 3 months. Three different *Agrobacterium* strains, LBA4404, EHA105, and AGLØ, were compared for efficiency in transformation. While differences in decontamination of co-cultivated cultures were observed among the different *Agrobacterium* strains, each produced putative transgenic lines from all tissues. The most productive tissues for putative transgenic lines were embryogenic calli. Putative transgenic lines were observed 5 weeks after co-cultivating embryogenic calli with *Agrobacterium* whereas all of the differentiated tissues developed selectively growing sectors 3 months after co-cultivation. Only 20% of the embryogenic callus clumps developed selectively growing sectors that continued growing the presence of the same or higher concentrations of the antibiotic G418 (geneticin). PCR amplification of the selection gene was conducted as additional evidence of genetic transformation. Reference: Chen, F.-C. and A.R. Kuehnle. 1996. Obtaining transgenic Anthurium through *Agrobacterium*-mediated transformation of etiolated internodes. J. Amer. Soc. Hort. Sci. 121:47-51.

P-1020

Transgenic *Medicago truncatula* Plants Obtained After Root and Hairy Root Transformation. CINDY CRANE and Zeng-Yu Wang. Forage Improvement Division, The Samuel Roberts Noble Foundation, Ardmore, OK 73401. Email: cjcrane@noble.org

Medicago truncatula is a forage legume that has been developed into a model legume. Large number of ESTs (>200,000) have been sequenced and the whole genome sequencing is in progress. Genetic transformation is still the bottleneck for testing gene functions in *M. truncatula*. As a model plant, *M. truncatula* is particularly useful for the study of root endosymbiotic associations, including nodulation and mycorrhizal colonization. An important requirement for such a model species is the possibility of introducing and analyzing chimeric transgenes in root tissues. After optimization of media compositions and *Agrobacterium* strains, we developed an efficient system for generating transgenic plants from *M. truncatula* root based on *A. tumefaciens*-mediated transformation. Furthermore, we developed a protocol capable of recovering transgenic plants from hairy root obtained by *A. rhizogenes*-mediated transformation. Transgenic nature of the recovered plants was confirmed by molecular analyses. The transgenic plants were fertile and produced seeds. This is the first report on generating transgenic *M. truncatula* plants by root and hairy root transformation.

P-1021

Efficient Transformation of Model Legume *Medicago truncatula* (Jemalong A17) by Using Cotyledon as Explant. ELANE WRIGHT and Zeng-Yu Wang. Forage Improvement Division, The Samuel Roberts Noble Foundation, Ardmore, OK 73401. Email: ecwright@noble.org

Medicago truncatula is an emerging model species for legumes because of its small diploid genome, self-fertility and short life cycle. Large number of ESTs (>200,000) have been sequenced and the whole genome sequencing is in progress. The ecotype Jemalong A17 has been used for sequencing of *M. truncatula*, therefore genetic transformation of A17 has attracted special interest. Unfortunately, the reported *in planta* system was not reproducible. It is known that A17 is a recalcitrant ecotype to regenerate and transform. We use cotyledon as explant for direct shoot formation, bypassing the callus formation stage. After optimization of a number of transformation parameters (*Agrobacterium* strains, media composition etc.), we developed an efficient transformation system for A17. A large number of transgenic plants can be regenerated and established in the greenhouse in a mere 3-4 months after transformation. Molecular analyses demonstrated transgenic nature of the regenerated plants. The transgenes were inherited to the progenies following Mendelian rules.

P-1022

Endosperm Morphology and Transcript Profiles of Storage Proteins in Developing Castor Seeds. GRACE Q. CHEN, Yeh-Jin Ahn, Xiaohua He, and Thomas A. McKeon. U.S. Department of Agriculture, Agriculture Research Service, Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710. Email: QHGC@pw.usda.gov

Castor oil is the only commercial source of ricinoleic acid and has numerous industrial applications, such as lubricants, coatings, plastics and fungicides. However, castor cultivation and processing generate highly hazardous seed storage proteins, toxin ricin and hyper-allergenic 2S albumins. Its seed meal needs to be heat-denatured after the oil extraction. The health concern and energy cost have thus limited castor domestic production. As a part of a genetic approach to eliminating ricin and 2S albumin from castor, we investigated their gene expression profiles during seed development. To assess seed development age quickly and accurately, we established a set of simple criteria, which included two visual markers, seed coat color and endosperm volume, and defined three phases that encompass the course of castor seed development. Northern analyses showed different temporal expression patterns between ricin and 2S albumin genes, indicating distinctive regulatory mechanisms involved in their mRNA accumulation. During the course of castor seed development, the expression of the 2S albumin was very low in early stages before the endosperm development. The mRNA levels peaked at middle stages when the endosperm underwent rapid growth. Once the endosperm tissue had fully expanded and entered maturation, the mRNA levels decreased. By comparison, no ricin mRNA was detected before the endosperm emerged. A significant amount of ricin mRNA was induced when the endosperm started expansion, and the expression increased aggressively during the rest stages of endosperm development until the maturation. Further characterization of the transcript profiles of 2S albumin from two castor cultivars indicated that only a single gene was expressed. Protein domain analysis revealed that castor 2S albumin contains the trypsin/alpha-amylase inhibitor pfam domain, suggesting a role for the albumin in insect resistance.

P-1023

Genetic Engineering of Cotton (*Gossypium hirsutum* L.) with Antifungal Proteins and Peptides to Confer Enhanced Resistance to Fungal Pathogens. KANNIAH RAJASEKARAN. USDA, ARS, SRRC, 1100 Robert E. Lee Blvd., New Orleans, LA 70124. Email: krajah@src.ars.usda.gov

Cotton crop is affected by several fungal and bacterial pathogens and the average annual cotton production loss due to diseases in the United States is about 12%. The seedling disease complex, fungal wilt pathogens and boll rots are the major cotton diseases worldwide. Cottonseed is also colonized by the saprophytic fungus, *Aspergillus flavus* that produces aflatoxin. Aflatoxin, one of the deadliest mycotoxins known, is produced by the fungus on other crops as well such as corn, peanuts and tree nuts. The presence of aflatoxin in cottonseed endangers the health of livestock consuming cottonseed meal and the health of humans consuming milk products from the affected livestock. To effectively eliminate or reduce aflatoxin levels in cottonseed to meet increasingly stringent regulatory levels, a variety of approaches will be necessary. Genetic engineering of cotton with antifungal proteins or peptides is an effective, viable and environmentally-safe option. We have transformed cotton with a gene encoding for a bacterial chloroperoxidase (*cpo-p*) or a synthetic antimicrobial peptide (D4E1) to provide resistance or tolerance to phytopathogens including *A. flavus*. These antifungal proteins have been shown to inhibit growth and development of pre-germinated conidia of *A. flavus*, *Fusarium* and other phytopathogens, including bacterial pathogens at low concentrations. Similar results were obtained earlier with the transgenic tobacco model system. Crude protein extracts from leaf tissue of transgenic tobacco plants expressing the CPO-P or the synthetic peptide D4E1 significantly reduced *in vitro* the number of fungal colonies arising from germinated conidia of *A. flavus*, *F. verticillioides* and *Verticillium dahliae* and showed greater levels of disease resistance *in planta* to the fungal pathogen, *Colletotrichum destructivum*, which causes anthracnose. In this presentation, we report on the development of transgenic cotton lines expressing the antifungal proteins with enhanced resistance *in vitro* and *in planta* to several fungal pathogens including *A. flavus*.

P-1024

Improved Production of Psoralen - a Bioactive Compound from In Vitro Grown Plants of *Psoralea corylifolia*. VEENA AGRAWAL and Satendra Singh. Department of Botany, University of Delhi, Delhi 110007, INDIA. Email: drveena_du@yahoo.co.in

Naturally occurring Psoralens, such as 8-Methoxypsoralen (8-MOP), 5-Methoxypsoralen (5-MOP), and substituted Psoralens have been identified as phytoalexins which are especially recommended in the treatment of leucoderma, leprosy, psoriasis, and inflammatory diseases of the skin. They are prescribed both for oral administration and for local external applications in the form of a paste or ointment (Anonymous, 1989). Psoralens are also used for determining the structure of both cDNA and RNA in viral, bacterial and mammalian systems including the role of small nuclear RNAs in processing heteronuclear RNA (Cimino *et al.*, 1985). Phytochemical evaluation of *in vivo* and *in vitro* plants has been carried out using HPLC for estimating Psoralen content. Quantitative analysis of Psoralen of different *in vivo* plant parts like leaf, node, root and entire plant have revealed that maximum quantity of Psoralen is present in roots (241.82 µg/g fresh wt.) followed by entire plant, leaf and nodal segment containing 165.0, 99.38, and 85.56 µg/g fresh wt. respectively. Similar to *in vivo* plant parts, maximum quantity of Psoralen was detected in roots (463.0 µg/g fresh wt.) where as the other plant parts *viz.* node, leaf, entire plant and embryonal axis derived plant contained 315, 227.84, 157.22 and 4.94 µg/g fresh wt. Psoralen, respectively. Interestingly the quantity of Psoralen was significantly higher in all the *in vitro* plant parts. Except entire plant, the quantity of Psoralen enhanced to almost double in roots and 3 to 4 times in leaf or node, respectively.

P-1025

An In Vitro Culture System with Isolated Spores and Transformed Carrot Roots to Study the Steps in the Life Cycle of an Arbuscular Mycorrhizal Fungus. G. NAGAHASHI. USDA, ARS, Eastern Regional Research Center, Wyndmoor, PA 19038. Email: gnagahashi@errc.ars.usda.gov

AM fungi are obligate symbionts which colonize host roots and make nutrients in the soil more available to the plant in exchange for fixed carbon. To complete the life cycle axenically, and *in vitro* culture system was used. The first interaction between fungus and host is the stimulation of hyphal elongation and branching by constitutive plant components. A rapid and sensitive bioassay was developed to test for soluble (root exudate components) and particulate root fractions (root caps and border cells) which stimulate the growth of the fungus during precolonization events. Secondary metabolites in the root exudate are the most prolific stimulators of hyphal branching of *Gigaspora gigantea* although the presence of activity in particulate fractions is also significant. So far, spore germination, hyphal growth and branching of the germinated spore, and appressoria formation have been completed in culture without a living host root.

P-1026

Transgenic Expression of Novel Purple Acid Phosphatase and Phytase Genes from *M. truncatula* Results in Improved Acquisition of Organic Phosphorus by *Arabidopsis*. Kai Xiao¹, Maria J. Harrison², and ZENGYU WANG¹. ¹Forage Improvement Division, The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, and ²Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, NY 14853-1801. Email: zywang@noble.org

Phosphorus (P) is one of the least-available nutrients in soils. Large amounts of P fertilizers are applied to cropland each year, but only 10% to 20% of the fertilizer P applied in soil is readily utilized by plants. Most of the total soil phosphorus (P) is not available for uptake due to its rapid immobilization by soil organic and inorganic components. We isolated a purple acid phosphatase (PAP) cDNA (*MtPAP1*) and a phytase cDNA (*MtPHY1*) from the model legume *Medicago truncatula*. Chimeric gene constructs were made in which the *MtPAP1/MtPHY1* were either driven by the root-specific MtPT1 promoter or by the constitutive CaMV35S promoter. The constructs also included either the native signal sequences of *MtPAP1/MtPHY1* or a patatin signal sequence from potato. Transgenic *Arabidopsis* plants carrying the chimeric *MtPAP1/MtPHY1* gene constructs showed 4.6- to 16-fold higher acid phosphatase or phytase activities in the root apoplast relative to the control plants. The increase in enzyme activity correlated closely with the expression levels of the transgenes. The expressed PAP or phytase was secreted into the rhizosphere as demonstrated by enzyme activity staining and HPLC analysis of phytate degradation by root exudates. Transgenic lines showed a minimum of a twofold increase in biomass production and total phosphorus content when phytate was supplied as the sole source of phosphorus. The results clearly demonstrate that transgenic expression of PAP or phytase genes of plant origin has great potential for improving plant phosphorus acquisition and for phytoremediation.

P-1027

Rapid, Scalable, Transient Protein Expression in Plant Cell Suspension Culture. W. R. CURTIS, J. I. Collens, K. M. O'Neill, H. S. Mason², L. B. Andrews, R. J. Hesser, and N. G. Sosale. Department of Chemical Engineering, The Pennsylvania State University, University Park, PA 16802 and ²School of Life Sciences, Arizona State University, Tempe, AZ 85287. Email: WRC2@psu.edu

A rapid protein expression platform for plants is described that permits production of milligram quantities of plant-expressed heterologous protein in a time frame of days without transformation and cell line selection. Gene transfer is facilitated using an *Agrobacterium tumefaciens* cysteine-auxotroph that was developed for controlled growth and T-DNA transfer in cell suspension culture. This system has been developed using the *gus*-intron system to allow for qualitative and quantitative screening of culture conditions that facilitate gene transfer. Initial levels of transient expression were very low (achieving 0.005% of total soluble protein [TSP]) despite comparing about a dozen different plant tissue types; *N. glutinosa* expression levels are much higher than *N. benthamiana* or *N. tabacum*. Synchronization of cell cultures by nutrient starvation has improved expression levels to 0.05% TSP, which is comparable to *Agrobacterium* leaf infiltration. Finally, engineering the plant tissues with inducible geminivirus 'replicase' in conjunction with a subgenomic viral vector has been tested. Although enhanced expression was observed that is consistent with viral-amplified T-DNA, the absolute levels are still much lower than was accomplished by screening non-amplifiable vectors against the replicase-containing transgenic plant lines (approaching 0.5% TSP). We have successfully implemented this transient expression system in an 80-L bioreactor where expression levels were superior to shake flask controls. Besides providing a means of producing plant-expressed therapeutic proteins, this transient expression system should be useful for other gene expression studies in plants.

P-1028

New Patent Laws and Regulations. STEVEN R. LUDWIG. Sterne, Kessler, Goldstein & Fox, PLLC, Washington, DC 20008. Email: sludwig@skgf.com

While working in the lab or at a desk, researchers often think of new ideas that could be patented. Although obtaining a patent is not inexpensive, many ideas are worth the investment. The investment often comes from an organization or corporation with the financial ability to cover the cost of obtaining a patent. This presentation will discuss how to persuade a university technology transfer office or a corporation to fund filing of a patent application by understanding what is patentable. A little bit of homework can go a long way in presenting a convincing argument that an idea is worth the investment. Any argument presented must reflect an understanding of patent law. Accordingly, recent case law and US Patent and Trademark Office regulations affecting the patenting of a researcher's ideas will be discussed. Also, the effect of the proposed U.S. post-grant opposition legislation on patent filings will be examined.

P-1029

European Regulation on Traceability and Labeling of Genetically Modified Food and Feed: Analysis on the Public Debate. L. MARTINELLI¹, F. Marin¹, E. Collavin², and G. Pellegrini². ¹Istituto Agrario San Michele all'Adige, 38010 San Michele all'Adige (TN), ITALY and ²Observa, ITALY. Email: Lucia.Martinelli@iasma.it

Answering to consumers' requests for information and safety concerns, European Union has recently released two laws on traceability and labeling of GM food and feed (CE 1829/2003, 1830/2003). With a qualitative research based on *focus group* methodology, we assessed in the Trento Province the level of agreement of the various actors involved in the matter. Two focus groups were devoted to stakeholders, while two involved citizens. Opinions were collectively validated, meetings were videotaped, and a DVD was produced. Our analysis verified a strong non-acceptance of GM products both in stakeholders and lay citizens. EU regulation on traceability and labeling was judged a promising tool toward an enhanced defense of consumer's right. However, while stakeholders admitted that their attitude arose from market motivation, citizens expressed concern based on a complex combination of emotional, political, cultural and ethical aspects, and appeared dazed on their own competence in label understanding. They also showed a strong feeling of outrage against GM technology applications. Our analysis confirmed the critical role of communication, which needs to be managed with extreme expertise. Thus, we believe that a more convinced attention by the scientific community to the communication aspects of risk management would enhance the debate level on agrobiotechnology applications and help the public decision-making process. Research supported by the Trento Autonomous Province, Project OSSERVA3.

I-2000

The Assessment of Roles for Arylphorin In Vivo and In Vitro. R. S. HAKIM^{1,2}, M. Blackburn², P. Corti^{2,3}, D. Gelman², C. Goodman⁴, K. Elsen⁵, M. Loeb², D. Lynn², and G. Smagghe^{5,6}. ¹Howard University, Dept. Anatomy, Washington, DC 20059; ²Insect Biocontrol Lab, USDA-ARS, Beltsville, MD 20705; ³University of Milan, Dept. Biology, 20133 Milan, ITALY; ⁴USDA-ARS, BCIRL, Columbia, MO 65203; ⁵Lab Cell Genetics, Vrije Universiteit Brussel, Brussels, BELGIUM; and ⁶Lab Agrozoology, Ghent University, Ghent, BELGIUM. Email: rhakim@mac.com

In insects, developmental responses are organ and tissue specific. As part of studying insect midgut cells in primary tissue cultures, both growth and differentiation factors have been identified from the growth media and its additions. Recently, arylphorin, a component of the fat body extract added to our cultures, was analyzed and determined to have a mitogenic effect on the stem cells of the tobacco budworm *Heliothis virescens* (Blackburn et al., Arch. Insect Biochem. Physiol, 55, 26-32, 2004). To get a broader understanding of the role of this protein, arylphorin and chymotrypsinized arylphorin was added to culture media for both primary and continuous cell cultures, and was evaluated for in vivo effects by addition to the diets of several insect species. The results demonstrate a mitogenic role in midgut primary stem cell cultures of several species, and with the enzymatically-treated material, on a fat body cell line. In vivo, arylphorin provided in the diet increases insect growth in almost all species tested.

I-2001

Effect of Plant Lectins on Growth of Insect Midgut Cells. G. SMAGGHE¹, J. Ryckaert^{1,2}, T. Soin¹, G. Caputo³, and E. J. M. Van Damme². ¹Lab Agrozoology, ²Lab Biochemistry and Glycobiology, Ghent University, Ghent, BELGIUM, and ³National Resources Canada, Canadian Forestry Service, Sault Ste Marie, Ontario, CANADA. Email: guy.smagghe@ugent.be

Feeding trials with insects have shown that some plant lectins provoke toxic effects or affect insect development and fecundity, which makes them putative candidates as insecticidal proteins. At present, the mode of action of lectins on insects is still poorly understood. However there is good evidence that plant lectins can interact with the insect midgut, and by doing so interfere with insect growth. In this study, we studied the effect of a series plant of lectins with different carbohydrate binding specificities on the growth of midgut insect cells, FPMI-CF-203, from the eastern spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). Spruce budworm is the most destructive native insect defoliator in northern spruce and balsam fir forests in Canada and the eastern United States. The activity of the plant lectins is discussed in relation to their carbohydrate binding properties. We also elaborate on the possible mode of action for the lectins in the insect midgut cells.

I-2002

Distribution of Elongation Factor-1 α in Larval Tissues of the Fall Armyworm, Spodoptera frugiperda. J. Habibi¹, C. L. GOODMAN², and M. K. Stuart³. ¹University of Missouri, Columbia, MO 65201-5297; ²USDA, ARS, Biological Control of Insects Research Laboratory, Columbia, MO 65203-3535 (CLG); and ³Still University of Health Sciences, Kirksville, MO 63501. Email: goodmanc@missouri.edu

Elongation factor-1 α (EF-1 α) promotes the delivery of aminoacyl-tRNA to the acceptor site of the ribosome during protein synthesis. It also plays a pivotal role in regulating apoptosis: cultured insect cells that accumulate EF-1 α become apoptotic, possibly by enhancing translation of "killer factors" such as caspase enzymes. Mab 7D6, a monoclonal antibody generated to EF-1 α from the fall armyworm, inhibits in vitro translation when added to lysates of Sf21 cells. Our long-term goal is to clone a single-chain antibody (scFv) gene encoding Mab 7D6 into the baculovirus to enhance the virus's potential as a biological control agent. We anticipate that fall armyworm larvae infected by the recombinant viruses will die more quickly than those infected by wild-type viruses. As Mab 7D6 binds to cytoplasmic EF-1 α , the antibody may prevent the synthesis of proteins vital to the host cell, or enhance virion production by delaying apoptosis. Because immunologically distinct, tissue-specific forms of EF-1 α commonly occur in eukaryotes, tissues of fall armyworm larvae were probed with Mab 7D6 to determine whether the tissues most important for establishing viral infection were recognized. Using western blotting, ELISA, and microscopy techniques, we found that all tissues examined contained measurable amounts of EF-1 α reactive with Mab 7D6, although concentrations varied among different cell types within a given tissue. The intensity of the signals was much stronger on the apical part of the columnar epithelial cells, especially on brush-border microvilli, than the basal parts of these cells. No signal was observed on goblet cells and basement membrane.

P-2000

Effects of Ethylene in Barley (*Hordeum vulgare* L.) Tissue Culture Regeneration. A. K. JHA,¹ L. S. Dahleen², and J. C. Suttle². ¹Department of Plant Sciences, North Dakota State University, Fargo, ND 58105 and ²USDA-ARS, Fargo, ND 58105. Email: ajay.jha@ndsu.edu

Ethylene is a gaseous plant hormone that regulates numerous cellular processes from germination to flowering and senescence. It is produced under stress conditions such as tissue culture and can be physiologically significant in-vitro due to enclosed conditions. This study was conducted to determine genotype-dependent ethylene production and its role in regeneration of barley (*Hordeum vulgare* L.) callus. Six barley cultivars were examined and found to produce different amounts of ethylene during culture. The highest regeneration was observed in cultivars generating the most ethylene. The media was modified by adding the ethylene precursor, ACC (1-amino-cyclopropane-1-carboxylic acid) or the ethylene antagonist silver nitrate (AgNO₃) to the media at different stages of callus culture to determine the effects of ethylene during plant regeneration. Further manipulation of ethylene synthesis and/or action will be used to identify critical times for ethylene effects on plant regeneration from recalcitrant genotypes and the results will be presented.

P-2001

Cryopreservation of *In Vitro* Grown Shoot Tips for Long-term Conservation of *Dioscorea* spp.: An Endangered Medicinal Plant. SONALI DIXIT SHARMA. Avestha Genegraine Technologies Private Limited, 9th floor, Discoverer, ITPL, Bangalore 560066, INDIA. Email: sonali-dixit@lycos.com

Dioscorea is an important medicinal plant commercially exploited for the extraction of diosgenin, a precursor of steroid drugs. Due to over exploitation and shrinking of natural habitat, this species which is native to India, faces a serious threat to extinction. Being vegetatively propagated, the species faces problems for its *ex situ* conservation using the conventional method of field maintenance. Thus, development of a cryopreservation protocol for *Dioscorea* spp. is especially desirable. Cryopreservation protocols have been developed and applied to cell cultures of various plant species to retain the capability of cultures for regeneration and production of secondary metabolites. However, shoot tips/meristems are ideal explants for *in vitro* conservation of plant diversity as plants regenerated from organized explants such as shoot-tips/meristems, usually maintain their genetic integrity. Employing the newly emerged techniques of vitrification as well as encapsulation-dehydration, successful cryopreservation of shoot tips of *in vitro* grown plantlets has been achieved with subsequent high frequency plant regeneration. The dissected shoot tips were cultured in preculture medium for 16 h before using for either of the techniques. For vitrification procedure, the precultured shoot tips were treated with loading solution followed by dehydration with plant vitrification solution (PVS 2, i.e., 15% DMSO, 30% glycerol and 15% ethylene glycol) and plunging in liquid nitrogen. Maximum survival and regeneration was obtained when the shoot-tips were treated with PVS2 at 0° C compared to that at 25° C. After rewarming, the shoot tips were treated with high sucrose unloading solution and plated on medium supplemented with plant growth regulators for recovery growth. During recovery growth, the cryopreserved shoot tips showed high frequency regeneration of shoots within 5 weeks after plating and subsequent proliferation of shoots. Likewise, for encapsulation-dehydration procedure, the precultured shoot tips were encapsulated in calcium alginate beads, pretreated in high sucrose solution (0.75 M), dehydrated in laminar airflow for 4 h and then plunged in liquid nitrogen. Both vitrification and encapsulation-dehydration techniques were found to be equally effective for cryopreservation of shoot tips. As these procedures are simple and technically less demanding, the routine use of these cryopreservation procedures for the *ex situ* long-term conservation of *Dioscorea* spp. will provide additional security before the species becomes extinct.

P-2002

Induction of Multiple Shoots and In Vitro Flowering in Soybean. YOUNG JIN KIM, Seung Bum Lee, and Henry Daniell. Department of Molecular Biology and Microbiology, University of Central Florida, 4000 Central Florida Boulevard, Biomolecular Science, Building 20, Room 336, Orlando, FL 32816-2364. Email: daniell@mail.ucf.edu

A successful, efficient system for multiple shoot induction, *in vitro* flowering and plant regeneration of soybean (*Glycine max*) was established. Three soybean genotypes were compared for organogenic responses on various media cultured under light conditions. Three-day-old cotyledons after germination induced adventitious shoots (94%, 4.7 shoots/cotyledon), directly from cotyledons by the hormone treatment and efficiency was higher than other conditions. In Pungsannamulkong cultivar (shoot formation rate, 99%) the MS medium was supplemented with 2.5 mg/l 6-benzylaminopurine (BAP). In Bosukkong cultivar (94%) the MS medium was supplemented with 1.5 mg/l thidiazuron (TDZ), but for Williams cultivar 82 (92%), MS medium supplemented with 0.5 mg/l TDZ, 1 mg/l BAP, and 0.01 mg/l α -naphthalene acetic acid (NAA), 3% sucrose, 0.4% phytigel and pH 5.8 was optimal. Plantlets were transferred onto the same medium supplemented with 1% activated charcoal for further development. With this treatment, regenerated plantlets were obtained within 8 weeks. *In vitro* induction of flowering was developed from auxiliary buds of mature cotyledon in soybean. Although the proportion of seedlings induced to flower is influenced by genotypic variation, a cytokinin or a shift in the auxin-cytokinin equilibrium is known to bring about *in vitro* induction of flowering. The optimal medium for the induction of *in vitro* inflorescence from cotyledon in Bosukkong (flowering induction rate, 74%) and Pungsannamulkong (63%) was MS medium supplemented with 2 mg/l TDZ. Some of inflorescence buds were developed and set seeds but we observed reduction of pollen fertility.

P-2003

Efficient *In Vitro* Regeneration System from Seed Derived Callus of Perennial (*Lolium perenne*) and Annual Ryegrass (*Lolium multiflorum*). SIVA CHENNA-REDDY, S. J. Allyson, D. A. Jennifer, S. L. Goldman, and R. V. Sairam. Plant Science Research Center, University of Toledo, Toledo, OH 43606. Email: srudrab@utnet.utoledo.edu

The commercially important ryegrasses in cool temperate climates throughout the world are annual (*Lolium multiflorum* L.) and perennial ryegrass (*Lolium perenne* L.). Improvements through conventional breeding in these ryegrasses have been slow as they are usually heterozygous and highly self-infertile. Hence, there is a need to use modern biotechnological tools to the development of improved rye grass cultivars for incorporating value added traits. Successful transformation of rye grasses has been done using suspension cells which is time consuming and laborious. We report here a rapid and highly efficient *in vitro* plant regeneration system from seed derived callus in annual (RGANN) and perennial rye grasses (RGPGR). After one week of seed germination in dark, the emerging shoot and root were chopped to suppress germination and stimulate callus formation and transferred to fresh medium. After 6 weeks, the resulting callus was maintained by sub-culturing every 4 weeks. For regeneration, cultures were incubated at 24 \pm 2° C under a 16/8-hour dark photoperiod. The treatment with 4.0 mg/l 2,4-D was found to be the best for callus induction for both RGANN and RGPGR and with further increase in 2,4-D concentration, the callus induction frequency decreased. Highest frequency of callus induction was observed in RGANN (94%) followed by RGPGR (72%) on MS medium supplemented with 4.0 mg/l 2,4-D. Callus initiation and growth took about 6 weeks. Highest frequency of shoots were regenerated per callus clump on MS +0.5 mg/l BAP in RGPGR (13.67). Whereas, in RGANN (Ryegrass), highest frequency of shoots (11.13) were regenerated in MS medium with 0.1 mg/l BAP. In both the varieties, a decrease in shoot regeneration frequency was observed in the combination treatments of BAP (0.1 mg/l, 0.5 mg/l) and 2,4-D (0.1 mg/l, 2.0 mg/l). Hundred percent rooting was observed when regenerated plants were transferred to MS media supplemented with 0.2 mg/l NAA alone or in combination with 0.5 mg/l GA3. Rooted plantlets were transferred to soil and acclimatized in the greenhouse.

P-2004

A Novel Genotype Independent Protocol for In Vitro Plant Regeneration from Mature Seed Derived Callus of Tall Fescue (*Festuca arundinacea* Schreb). SIVA CHENNAREDDY, T. V. Reddy, S. L. Goldman, and R. V. Sairam. Plant Science Research Center, University of Toledo, Toledo, OH 43606, Email: srudrab@utnet.utoledo.edu

Tall fescues (*Festuca arundinacea* Schreb.) are cool season forage and turf grasses of significant agricultural importance in different grassland countries. Genetic improvement of tall fescues by conventional selection procedures is slow, since these are predominantly, cross-pollinated, hexaploid and generally infertile. Genetic engineering approaches for incorporation of agronomically useful traits may contribute to the development of improved tall fescue cultivars. We report here a rapid and efficient *in vitro* plant regeneration system from mature seed derived callus of two Tall fescue cultivars (FESEVB- EnviroBlend and FESEVS-EnviroSHADE) on solid medium which can be exploited for incorporating genes conferring value added traits. The treatment with 4.0 mg/l 2,4-D was found to be the best for callus induction for both the varieties and further increase in 2,4-D concentration decreased the callus induction frequency. Highest frequency of callus induction was observed in FESEVS (88%) followed by FESEVB (86%) on MS medium supplemented with 4.0 mg/l 2,4-D. Callus was maintained every 4 weeks on MS medium containing 4.0 mg/l 2,4-D and 0.1 mg BAP. Callus turned greenish and shoots appeared after 4 weeks of transfer to regeneration medium. Higher frequency of shoots were regenerated per callus clump on MS + 0.5 mg/l BAP alone compared to other hormonal combinations in FESEVB (13.07) followed by FESEVS (11.13). When 2, 4, D was present in regeneration medium a drastic reduction in shoot regeneration frequency was observed. Rooting was observed in two weeks after transferring the regenerated plants to the rooting medium. Hundred percent rooting was observed in both the varieties on MS media supplemented with 0.2 mg/l NAA. In the combination treatments of NAA and GA₃ all the plants were rooted in FESEVS while only 80% of the plants rooted in FESEVB. When BAP (1.0 mg/l) was added to the rooting medium, either with NAA alone or in combination with NAA and GA₃, a drastic reduction in rooting frequency was observed. All rooted plants were transferred to the soil and acclimatized in the greenhouse.

P-2005

Evaluation of Chloroplast Derived Cholera Toxin B Subunit (CTB) and Green Fluorescent (GFP) Fusion Protein for Oral Delivery. BRITTANY E. BURNS, Arati-Ulhas Limaye, and Henry Daniell. Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science Building 20, Room 336, Orlando, FL 32816-2364. Email: daniell@mail.ucf.edu

Many infectious diseases require booster vaccinations or multiple antigens to induce and maintain protective immunity. Advantages of plant-derived vaccines include the delivery of multiple antigens, low cost of production, storage & transportation, elimination of medical personnel and sterile injections, heat stability, antigen protection through bioencapsulation, the generation of systemic & mucosal immunity and improved safety via the use of a subunit vaccine and absence of human pathogens. In an effort to study the oral delivery of therapeutic proteins using the transmucosal carrier CTB, a fusion of CTB-smGFP was expressed in transgenic chloroplasts of *Nicotiana tabacum* var. petit Havana by inserting the CTB and smGFP genes into the chloroplast genome. The pLD-CTB-smGFP vector contains CTB with a C-terminal fusion to smGFP separated by a furin cleavage site. Both genes were inserted into a universal chloroplast vector, pLD-ctv containing the 16S rRNA promoter, the aadA gene coding for spectinomycin selectable marker gene, the psbA 5' & 3' untranslated regions to enhance translation in the light and trnI, trnA homologous flanking sequences for site specific integration into the chloroplast genome. Chloroplast integration of the CTB-smGFP genes was confirmed by PCR and Southern blot analysis. The CTB-smGFP fusion protein expression was confirmed by smGFP expression under UV light and immunoblot analysis. Expression levels were quantified by ELISA. GM1-ganglioside binding assays confirmed that the chloroplast-derived CTB binds to the intestinal membrane receptor of cholera toxin, confirming correct folding and disulfide bond formation of CTB pentamers within transgenic chloroplasts. Functional studies are being carried out in mice to investigate the concept of bioencapsulation by plant cells by using smGFP as a visible marker as well as to test the ability of chloroplast-derived CTB to act as a transmucosal carrier of a reporter gene product. These investigations might facilitate the development of a novel cost effective oral delivery system for vaccines and therapeutic proteins.

P-2006

Expression of a Cholera Toxin B Subunit-rotavirus Enterotoxin Fusion Gene in Transgenic *Nicotiana*. ANILA KALLURI, William H. R. Langridge, and Henry Daniell. ¹Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science, Bldg. #20, Room 336, Orlando, FL 32816-2364 and ²Center for Molecular Biology and Gene Therapy, 161, Mortensen Hall, Loma Linda University, Loma Linda, CA 92350. Email: daniell@mail.ucf.edu

Rotaviruses are the single most important cause of virus-based acute diarrheal illness in infants and young children in industrialized and developing countries. The identification of a rotavirus nonstructural protein gene (NSP4) encoding a peptide, which functions both as a viral enterotoxin and as a factor involved in acquisition of a host cell membrane during virus budding from cells, provides a new approach for mucosal immunization. Transgene expression via the chloroplast genome offers a number of advantages including high level of expression and transgene containment via maternal inheritance. To achieve an enhanced immune response to rotavirus infection, a fusion gene encoding the cholera toxin B subunit linked to rotavirus enterotoxin 90 aa protein (CTB-NSP4₉₀) was used. The CTB-NSP4 fusion gene was cloned into pCR2.1 vector containing the psbA 5'UTR, that enhances translation during illumination. The 5'UTR-CTB-NSP4 gene cassette was finally cloned into the tobacco universal vector pLD-ctv between the trnA and trnI flanking region, sites for homologous recombination into the chloroplast genome. The final vector pLD-AK-CTB-NSP4 was bombarded into *Nicotiana tabacum* var. Petit Havana, and *Xanthi* leaves to generate transgenic chloroplast containing plants. Chloroplast integration of the CTB-NSP4₉₀ fusion gene was confirmed in transgenic tobacco plants by PCR analysis using 3P/3M primers. The integration of aadA, 5'UTR-CTB-NSP4 was additionally confirmed using the 5P/2M primer pairs. Southern blot analysis confirmed site-specific gene integration and homoplasmy. Immunoblot analysis of transformed chloroplasts confirmed expression of the CTBNSP4₉₀ fusion protein. Quantification of CTB-NSP4 fusion protein in transformed tissues by ELISA is in progress. Antibody titration and virus challenge experiments will be performed in mice to evaluate the antigenic and protective properties of the chloroplast derived CTB-NSP4 fusion protein.

P-2007

Expression of Soluble Modified Green Fluorescent Protein and Interferon Alpha-5 Fusion in Transgenic Chloroplasts. BRITTANY E. BURNS and Henry Daniell. Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science Building 20, Room 336, Orlando, FL 32816-2364. Email: daniell@mail.ucf.edu

The World Health Organization estimates that approximately 170 million people worldwide are infected with hepatitis C virus (HCV), with 3-4 million new cases each year, and that more than one third of the world's population is infected with hepatitis B virus (HBV). A large majority of HCV-infected patients have severe liver cirrhosis and currently there is no vaccine available for this disease. In addition, the rising cost of treatment for severe illnesses calls for the more economical production of therapeutic proteins. Alpha interferons have therapeutic uses, such as the inhibition of viral replication and cell proliferation, enhancement of the immune response, and most recently, the treatment of patients suffering from HCV. The Food and Drug Administration approved a specific subtype of interferon- α (IFN α 2b) for the treatment of HCV. In an effort to produce another subtype of interferon- α (IFN α 5, kindly provided by Dr. Jesus Prieto, Universidad De Navarra, Pamplona, Spain) in large quantities and free of contaminants for possible treatment options and oral delivery of HCV, a fusion of smGFP-IFN α 5 has been expressed in transgenic chloroplasts of *Nicotiana tabacum* var. dark fire, by inserting the smGFP (745 bp) and IFN α 5 (515 bp) genes into the chloroplast genome by homologous recombination. The pLD-BB1 vector contains smGFP with a C-terminal fusion to IFN α 5 containing a furin cleavage site between the fusion proteins. The genes were cloned into a universal chloroplast vector, pLD-ctv containing the 16S rRNA promoter, aadA gene coding for the spectinomycin selectable marker, psbA 5' & 3' untranslated regions to enhance translation in the light and trnI & trnA homologous flanking sequences for site specific integration into the chloroplast genome. Chloroplast integration of the smGFP-IFN α 5 genes was confirmed by PCR and Southern blot analysis. The smGFP-IFN α 5 fusion protein expression was confirmed by immunoblot analysis and smGFP expression under UV light. Expression was quantified by ELISA. The smGFP-IFN α 5 fusion protein is being further analyzed via *in vivo* studies. The expression of smGFP-IFN α 5 transgenic chloroplasts might facilitate the development of a new and alternate treatment for HCV and possible oral delivery options with a lower cost of production.

P-2008

Efficacy of Chloroplast Derived Lethal Factor-protective Antigen Fusion Protein as a Vaccine Against Anthrax. WILLIAM W. WONG, and Henry Daniell. Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science Building 20, Room 336, Orlando, FL 32816-2364. Email: daniell@mail.ucf.edu

The spores produced by *Bacillus anthracis* are the causative agent of anthrax. The Centers for Disease Control have listed anthrax as a category A agent in regards to its known bioterrorism potential. The current vaccine has contaminants (that result in negative reactions), requires multiple injections, annual boosters, and is in limited supply. These factors demonstrate the need for an improved vaccine. The edema and organ failure seen in anthrax patients is caused by the action of three individually non-toxic proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF). The 83 kDa immunogenic *Bacillus anthracis* PA has been successfully expressed in transgenic tobacco chloroplasts and shown to be highly immunogenic. In an attempt to further enhance the efficacy of this vaccine antigen, a fusion protein of LF devoid of toxin gene (LF 27) and PA 83 will be used to maximize the immune response. The pLD-LF27-PA83 containing the LF27-PA83 gene was cloned into a universal chloroplast vector, pLD-ctv containing the 16S rRNA promoter, aadA gene coding for the spectinomycin selectable marker, *psbA* 5' & 3' untranslated regions to enhance translation in the light and *trnI* & *trnA* homologous flanking sequences for site specific integration into the chloroplast genome. Western blots done on the completed chloroplast vector showed production of the PA fusion protein. Leaves from *Nicotiana tabacum* cv. Petit Havana were bombarded using the biolistic PDS 1000/He device. Spectinomycin resistant clones were produced, and subjected to two rounds of selection. The *in vitro* macrophage lysis assays, subcutaneous immunization, toxin neutralization and toxin/spore challenge of immunized animals (with purified chloroplast-derived LF27-PA83 fusion) will be performed to test functionality and efficacy. The high expression levels of LF27-PA83 and strong immune response may facilitate development of a safe and efficient anthrax vaccine at a lower cost of production.

P-2009

Expression of Hepatitis C Virus Non Structural 3 Antigen in Transgenic Chloroplasts. ANUBHUTI BHATTI¹, Juan Jose Lasarte², and Henry Daniell¹. ¹Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science, Bldg. #20, Room 336, Orlando, FL 32816-2364 and ²Universidad de Navarra, Facultad de Medicina Interna, Irunlarrea 1, 31018 Pamplona, SPAIN. Email: daniell@mail.ucf.edu

Hepatitis C virus infection is the major cause of acute hepatitis and chronic liver disease. An estimated 180 million people are infected globally (WHO). There is no vaccine available to prevent hepatitis C and treatment with antiviral drugs is expensive and is accompanied with various side effects. Therefore, there is an urgent need for the development of effective vaccine antigens and an efficacious HCV vaccine. The non-structural 3 protein of the hepatitis C virus is one of the most conserved and multifunctional protein of the virus and therefore is a good candidate for the development a HCV vaccine. Vaccine antigen production via chloroplast transformation system usually results in high expression levels and eliminates the possibility of contamination with viral vector sequences, human or animal pathogens. To express the HCV NS3 antigen in the chloroplast of *Nicotiana tabacum* var. Petit Havana and LAMD-609, the NS3 gene (1.9 kb) was cloned into a chloroplast expression vector, pLD-ctv containing the 16S rRNA promoter, aadA gene coding for the spectinomycin selectable marker, *psbA* 5' & 3' untranslated regions to enhance translation in the light and *trnI* & *trnA* homologous flanking sequences for site specific integration into the chloroplast genome. Chloroplast integration of the NS3 gene was first confirmed by PCR. Southern blot analysis further confirmed site-specific gene integration and homoplasmy. The NS3 protein was detected in transgenic chloroplasts by Immunoblot analysis. The NS3 protein was further quantified by ELISA. Maximum expression levels of NS3 up to 2% in the total soluble protein were observed even in old leaves, upon 3-day continuous illumination. These results demonstrate successful expression of the HCV non-structural 3 antigen in transgenic tobacco chloroplasts. Animal studies will be performed in Dr. Juan Jose Lasarte's laboratory, Universidad De Navarra, Pamplona, Spain.

P-2010

Expression of Cholera Toxin B Subunit-proinsulin Fusion Protein in Transgenic Tobacco Chloroplasts as a Treatment Against Development of Type-1 Autoimmune Diabetes. ANDREW L. DEVINE, William W. Wong, Arati Limaye, and Henry Daniell. Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science Building 20, Room 336, Orlando, FL 32816-2364. Email: daniell@mail.ucf.edu

Oral administration of disease specific autoantigens have been demonstrated to delay or even prevent the onset of disease symptoms, referred to as tolerance. We have produced a *Nicotiana tabacum* cv. petit Havana chloroplast transgenic lines that expresses a cholera toxin B subunit (from *Vibrio Cholerae*)-human proinsulin (a and b chain) fusion protein, designated CTB-Pris. This approach has been previously demonstrated by nuclear expression in potato tubers, to prevent the onset of insulin-dependent diabetes mellitus (IDDM) in NOD mice, when delivered orally. The pLD-PW contains the CTB-Pris gene cloned into the universal chloroplast transformation vector pLD-ctv in which the 16S rRNA promoter drives the aadA gene selectable marker, which confers resistance to spectinomycin; the *psbA* 5' untranslated region (UTR) enhanced translation of CTB-Pris in the presence of light and the *psbA* 3'UTR conferred transcript stability. The *trnI* and *trnA* homologous flanking sequences facilitated site-specific integration of transgenes into the tobacco chloroplast genome. Site-specific integration was demonstrated by PCR and Southern blot analysis with probes for both CTB and Pris. Western Blot analysis has demonstrated the presence of abundant CTB-Pris in transgenic plants with both CTB polyclonal and proinsulin monoclonal antibodies. Southern blot analysis has also confirmed that homoplasmy had been achieved in the T0 generation. These chloroplast transgenic lines grew slowly although their appearance was normal. Quantification studies are currently underway, which will be followed by animal studies on NOD mice in order to determine the ED50 for prevention of the onset of insulin-dependent diabetes mellitus.

P-2011

Selection of Salt Tolerant Plantlets from the Tissue Culture of Watermelon. S. A. FAROOQ and Talat Farooq¹, Department of Biology, Sultan Qaboos University, P O Box 36, PC123, OMAN. ¹Shadan Post Graduate Center, Hyderabad, INDIA. Email: sfarook@squ.edu.om

Protocols for *in vitro* shoot regeneration from cotyledon explants of watermelon (*Citrullus lanatus* (Thunb.) were standardized in two cultivars viz., Sugar Baby and Glory Jumbo using MS media supplemented with BA + IAA and BA + 2iP. Response of the cultures to NaCl were studied by adding 50 mM, 100 mM, 200 mM, 300 mM and 400 mM of Sodium Chloride (NaCl) to the media. Effect on the growth, shoot organogenesis, rhizogenesis and plantlet regeneration were studied. Significant decrease in fresh weight of callus, shoot bud formation and regeneration was noted. Higher levels of Proline accumulation was found in the callus and regenerated plantlets after exposure to different levels of NaCl. Attempts were made to isolate salt tolerant plantlets. Acclimatization has been taken up by growing salt tolerant plantlets in pots supplying nutrient solution supplemented with higher salt concentration.

P-2012

Monitoring Gene Expression Profiles During Cold Acclimation in Blueberry Under Field and Cold Room Conditions Using cDNA Microarrays. A. L. Dhanaraj, N. W. Alkharout¹, H. S. Beard¹, I. B. Chouikha¹, B. F. Matthews¹, and L. J. ROWLAND. Fruit Laboratory, ¹Soybean Genomics and Improvement Laboratory, Plant Sciences Institute, Beltsville Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705. Email: rowlandj@ba.ars.usda.gov

Environmental stresses, including low temperature extremes, reduce crop yields and impact the profitability and competitiveness of U.S. producers. The U.S. is the world's leading blueberry (*Vaccinium* spp.) producer. The blueberry industry in the U.S., however, suffers from a lack of winter hardy and spring-frost resistant cultivars. Our laboratory has been working toward increasing our understanding of the genetic control of cold hardiness in blueberry to ultimately use this information to develop more cold hardy cultivars. Here, we report using cDNA microarrays to monitor changes in gene expression associated with cold acclimation in blueberry under field and cold room conditions. Approximately 2400 cDNA clones were picked from two cDNA libraries that were constructed using RNA isolated from cold acclimated floral buds (collected in mid-winter) and non-acclimated floral buds (collected in fall) of the fairly cold hardy cultivar Bluecrop. 5' ESTs were generated from the cDNAs, assembled into contigs, and categorized based on BLAST search results. cDNA inserts were amplified from the plasmids, purified, and arrayed onto glass slides. Expression was monitored at multiple times during cold acclimation of plants under both field and cold room conditions. A large percentage of gene transcripts were found to be up-regulated during cold acclimation and, interestingly, more transcripts were found to be up-regulated under cold room conditions than under field conditions. These results suggest that plants may be responding to more stresses in the cold room than in the field environment.

P-2013

Engineering the Environmental Stress Regulator of Bahiagrass (*Paspalum notatum* Flugge). V. A. James and F. ALTPETER. University of Florida - IFAS, Agronomy Department, PMCB, Laboratory of Plant Molecular Physiology, 2191 McCarty Hall, Gainesville, FL 32611. Email: faltpeter@ifas.ufl.edu

Bahiagrass is an important turf and forage grass in the Southern US and in the subtropical regions around the world. The objective of this experiment was to further enhance the productivity and persistence of bahiagrass during seasonal periods of drought and / or freezing and in salt affected regions by over-expression of the stress inducible transcription factor CBF3. Transcription factors like CBF3 are capable of activating the expression of multiple genes involved in protection against environmental stresses (Kasuga et al., 1999). The CBF3 gene, HVA1 or Dhn8 promoter candidates were isolated from genomic wild or cultivated barley DNA by PCR. Primers for isolation of target genes were designed according to the published cultivated barley sequences. Plant transformation vectors were constructed on basis of vector pJFnpTII (Altpeter et al., 2000). Biolistic gene transfer was carried out 6 weeks after initiation of callus cultures from mature seeds. Transgenic plants expressing the selectable *nptII* gene were regenerated on paromomycin containing medium and confirmed with NPT II-ELISA (Agdia) (Altpeter and James, 2005). Transgenic plants over-expressing CBF3 are currently identified by real time RT-PCR and will be subjected to cold stress in a completely randomized block design in a controlled environment chamber. Leaf tissue damage will be visually scored 1 day after cold stress and biomass production will be evaluated four weeks after recovery from cold stress. Data correlating CBF3 over-expression in transgenic bahiagrass with freezing stress response will be presented. **References:** Altpeter, F., J. Xu, & S. Ahmed. (2000). Generation of large numbers of independently transformed fertile perennial ryegrass (*Lolium perenne* L.) plants of forage- and turf-type cultivars. *Mol. Breeding* 6:519-528. Altpeter, F. & V. James (2005) Genetic transformation of turf-type bahiagrass (*Paspalum notatum* Flugge) by biolistic gene transfer. *Intern. Turfgrass Soc. Res. J.* (accepted for publication). Kasuga, M., Q. Liu, S. Miura, K. Yamaguchi-Shinozaki, and K. Shinozaki. 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature Biotech.* 29:287-291.

P-2014

The Effect of *Myo*-inositol Concentration on Pinitol Levels in *Inositol Methyl Transferase (imt)*-containing Transgenic Embryonic Soybean. J. M. CHIERA, J. G. Streeter, C. A. Nemes, and J. J. Finer. Department of Horticulture and Crop Science, OARDC/The Ohio State University, Wooster, OH 44691. Email: chiera.4@osu.edu

Increased pinitol concentration in plants has been associated with increased drought and salinity tolerance. Pinitol is produced through the two-step conversion of *myo*-inositol. First, *myo*-inositol is converted to ononitol by inositol methyl transferase (IMT) and then to pinitol by an epimerase. In a previous experiment, we constitutively-expressed IMT from ice plant in soybean embryonic cultures and observed increased ononitol levels. Although over-expression of IMT effectively increased ononitol levels in embryonic cultures, pinitol concentrations in developing embryos were marginally increased. An inverse correlation between ononitol and *myo*-inositol levels was observed indicating *myo*-inositol might be limiting for subsequent pinitol production. To test this hypothesis *myo*-inositol was supplied at various concentrations in the media used for proliferation of the transgenic *imt*-containing soybean tissue. A positive correlation between *myo*-inositol and ononitol was observed in transgenic clones suggesting that the availability of *myo*-inositol can limit ononitol production. In "Jack" controls ononitol was not detected. During embryo development, pinitol concentrations in *imt*-containing clones increased while ononitol levels decreased suggesting the presence of the endogenous pinitol producing enzymes. In developing "Jack" embryos ononitol and pinitol were not detected.

P-2015

Transient Expression in Plant Cell Suspension Cultures Using an Auxotrophic Form of *Agrobacterium* is Shown to be as Effective as *Agrobacterium* Infiltration in Plant Leaves. L. A. ANDREWS, K. M. O'Neill, N. G. Sosale, J. I. Collens, and W. R. Curtis. Laboratory of Dr. Wayne Curtis, Department of Chemical Engineering, Pennsylvania State University, State College, PA 16802. Email: lba22@cornell.edu

Transient gene expression is a technique of achieving a rapid burst of gene expression without integrating the foreign DNA into the plant chromosome. Although *Agrobacterium* is used to make transgenic plants, it can also deliver T-DNA to the plant nucleus for transient expression during the period of several days after plant tissue infection. In this study, transient expression of the *gus*-intron reporter gene was facilitated in three different tobacco species. Two different approaches to T-DNA delivery were compared: 1) infiltration of a prototrophic strain of *Agrobacterium* into leaves, and 2) co-culture of plant cell suspension cultures with an *Agrobacterium* auxotroph. In carrying out this work, numerous improvements to both techniques were accomplished. Very poor expression was observed for agro-infiltration in aseptic tissue cultured plants; whereas, contamination (including associated extraneous GUS activity) was observed in garden-grown plants. Wounding of plant tissues with a wire brush prior to infiltration had a large positive impact on *N. benthamiana* leaves, but not for *N. tabacum* or *N. glutinosa*. The best expression level achieved by leaf infiltration was in *N. benthamiana* (0.025% total soluble protein). Cell suspension cultures were shown to be highly dependent upon the media used for culture, and the timing of subculture relative to the introduction of the *Agrobacterium*. A cell suspension culture line of *N. glutinosa* achieved and expression level greater than 0.04% TSP. In addition to providing comparable levels of expression, the cell suspension culture technique avoids contamination and provides highly controllable aseptic culture conditions to facilitate improvements in expression by control of the plant cell culture and *Agrobacterium* pre-culture and co-culture environments.

P-2016

Development of Plant Regeneration and Genetic Transformation in the Cotton. D. D. I. Grigina, M. MUMINOVA, and S. Djataev. Laboratory of Plant Biotechnology, Institute of Genetics, Tashkent, UZBEKISTAN. Email: biotech@uzsci.net

Induction of morphogenic response is one of general problem in tissue culture of cotton. In this case an efficient plant regeneration system of wild cotton species has been established. Of the eight wild genotypes (*Gossypium klotzchianum* And., *G. africanum*, *G. biki*, *G. anomalum*, *G. harknessii*, *G. tomentosum*, *G. raimondii*, and *G. lobatum*) were evaluated. Callus tissue was induced from leaf of perennial greenhouse plants. Genotypes, phytohormones combinations and concentrations determined differentiation embryos and formation tracheids in the callus. The leaf segments of *G. klotzchianum* And. produced embryogenic callus on MS medium supplemented with 2 mg/l NAA, 0.1 mg/L 2,4-D, and 1 mg/l kinetin. Embryos of various development stages (globular, heart and torpedo shaped) were observed. Large numbers of green plants were regenerated from the established embryogenic callus on the induction medium (IM) containing 10 mg/l 2iP. Agrobacterium-mediated transformation of leaf segments and embryogenic calli were optimized. Explants infection with GFP gene were sub-cultured on IM medium. Embryogenic callus expressing GFP genes are being recovered. Experiments on natural sensitivity and selection of transformed tissue under selective agents and PCR analysis of transgenic material will be presented.

P-2017

Production of Recombinant Human Serum Albumin in Transgenic Plant Suspension Cell Cultures. MELTEM MAVITUNA¹, Rainer Fischer², and Stefan Schillberg³. ¹University Hospital Aachen, Institute for Clinical Chemistry, Pauwelstrasse 30, 52074 Aachen, GERMANY; ²RWTH Aachen, Institute for Molecular Biotechnology, Worringerweg 1, 52074 Aachen, GERMANY; and ³Fraunhofer-Institute for Molecular Biology and Applied Ecology, Worringerweg 1, 52074 Aachen, GERMANY. Email: mmavituna@ukaachen.de

The utilization of plant cells for the production of natural or recombinant compounds of commercial interest has gained increasing attention over the past decades. In particular, the capability of plant cells to synthesize, process and target large complex mammalian proteins in a manner very similar to their natural hosts makes them an attractive alternative for recombinant protein production. Further benefits of using plants are the reduced technical, ethical and safety issues and costs compared with mammalian cell cultures or transgenic animals. When clinical use of recombinant proteins is intended, their production under defined, controllable and sterile conditions with straightforward purification protocols may be advantageous. Therefore, in this study we expressed human serum albumin (HSA) in transgenic *Nicotiana tabacum* cv. Bright Yellow 2 (BY-2) suspension cells. BY-2 cells were transformed by co-cultivation with recombinant *Agrobacterium tumefaciens*. Pinpoint-sized clumps of kanamycin-resistant cells were screened for expression of the chimeric gene and highest recombinant HSA producers were selected for the initiation of cell suspension lines. Once a rapidly growing homogeneous suspension culture was obtained, both the culture medium and the cell pellet were analysed for the presence of recombinant HSA. The transgenic cell line HSA-APO29 showed the highest accumulation level reaching 1.87 µg per gram cell material at day 7 upon sub-culturing. Intact recombinant HSA was efficiently secreted to the culture medium accumulating to 1-1.5 µg per ml. The use of plant cell suspension cultures for the production of recombinant proteins is still in its infancy. Further investigation of the culture conditions will be helpful to increase the yield and decrease the costs of this production system.

P-2018

Plastids Transformed: Expressing Microbial Resistance Genes from the Chloroplast Genome. T. A. RUHLMAN^{1,2}, K. Rajasekaran², and J. W. Cary². ¹Department of Biological Sciences, University of New Orleans, New Orleans, LA and ²USDA-ARS, Southern Regional Research Center, New Orleans, LA 70124. Email: truhlman@srrc.ars.usda.gov

While genetic improvement of susceptible crop species may enhance resistance to microbial pathogens and facilitate reduced pesticide load, the possibility for transmission of novel genes to wild relatives has hampered acceptance of GM crops in some markets. Chloroplast transformation presents an attractive alternative to nuclear transformation and offers the potential to ameliorate environmental concerns. Most agronomically important species exhibit maternal inheritance of organellar genomes eliminating the threat of transgene escape through pollen and gene silencing is absent. The border sequences in our vector, pLD-CtV, direct single copy insertion by homologous recombination in the inverted repeat region of the tobacco plastome. Each transformant contains up to 20000 copies of the transgene in a leaf cell, proteins can accumulate to high levels (up to 50% of total soluble protein) and are retained within the chloroplast envelope protecting them from degradation by host cytoplasmic proteases. Two antimicrobial gene constructs, a) a bacterial chloroperoxidase (*cpo-p*) and b) D4E1, a synthetic, lytic peptide, were ligated into pLD.CtV for transformation experiments. Transformants were identified by selection on regeneration media with 500 mg L⁻¹ spectinomycin, resistance to which was conferred by the aminoglycoside-3'-adenyltransferase (*aadA*) gene in the plasmid vector. PCR and Southern analysis has confirmed integration in the plastome. Western analysis confirms the presence of the CPO-P in higher abundance in chloroplast transformants than nuclear. Although integration and expression was achieved in these experiments, concomitant increase in the level of protein accumulation was not observed. Northern analysis of primary transformants for *cpo-p* showed 15 fold higher transcript abundance than nuclear transformants; yet this was not reflected in Western blot, enzyme or bioassay. In an effort to enhance the rate of translation a new construct was developed replacing the single ribosome binding site between *aadA* and *cpo-p* with the tobacco full length psbA 5' UTR.

P-2019

Agrobacterium-mediated Transformation of Maize Hi-II B Using Non-super-binary Vectors. Angie Kennon, Heather Temple, and ZHANYUAN ZHANG. Department of Agronomy and Plant Transformation Core Facility, Division of Plant Sciences, University of Missouri-Columbia, Columbia, MO 65211. Email: zhangzh@missouri.edu

Maize elite inbred lines are highly desirable for their economical traits as well as a lesser degree of heterogeneous genetic makeup. It is these characteristics that make transformation of maize elite inbred lines critical for maize genetic improvement and functional analysis of maize genes. Despite previous efforts made among various transformation laboratories, maize inbred transformation using non-super-binary vectors still suffers low transformation efficiency. Therefore, we have made efforts in developing efficient *Agrobacterium*-mediated inbred transformation using the Hi-II B maize genotype as a near-inbred line. Hi-II B was developed previously from elite inbred B73 by backcrossing with A188 for major regeneration QTLs (Armstrong et al., 1992, Theor. Appl. Genet. 84:755-762) and presented efficient regeneration responses with predominant type II embryogenic calli. We have further improved the embryogenic response of this line using an optimal level of L-proline during the callus initiation stage on N6 medium. In addition, we have further modified previously described protocols for maize transformation (Frame et al., 2002, Plant Physiol. 129:13-22; Zhao et al., 2004, U.S. patent 6822144). For efficient T-DNA delivery, we employed antioxidants L-cysteine and dithiothreitol (DTT) as additional critical medium components in combination with a low salt concentration during the co-cultivation period. The *Agrobacterium* strain EHA101 carrying non-super-binary vector pZY102 (also named PTF102) was used throughout the experiments. The pZY102 carries within its T-DNA a *bar* gene as a selectable marker and an intron GUS gene as a reporter. After 3-days co-cultivation, the embryogenic calli were first cultured on resting medium without selection for 7 days and then selected on N6 bialaphos-containing media. Bialaphos-resistant clones were carried over to the greenhouse and seed stage. We plan to analyze representative samples of bialaphos resistance T₀ plant lines and a few sets of progeny plants (T₁) using GUS assay and Southern blot analysis. Our preliminary results indicate that the Hi-II B is a good maize near-inbred genotype for *Agrobacterium*-mediated transformation. We will present more detailed experimental design, transformation protocol, data analysis, and transformation efficiencies.

P-2020

Novel Isoflavone C-glycosides Elicited in *Pueraria lobata* (kudzu) Cell and Root Cultures. N. ADAM REPPERT¹, M. A. Lila¹, Jeevan K. Prasain², Kenneth Jones², Ray Moore², and Stephen Barnes². ¹College of Natural Resources and Environmental Sciences, University of Illinois Urbana-Champaign, 1115 Plant Sciences Lab, 1201 S. Dorner Dr., Urbana, IL 61801 and ²Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL 35294. Email: reppert@uiuc.edu

In vitro cultures of *Pueraria lobata* (kudzu) were evaluated as a source of isoflavone C-glycosides, which have superior stability relative to isoflavone O-glycosides, and therefore have potential benefits for glucose homeostasis. Sterile *P. lobata* plantlets were grown in vitro, and leaf- and root-derived solid callus cultures were generated on a B5 medium (supplemented with 1 mg/ml 2,4-D, 1 mg/ml NAA, and 0.5 mg/ml kinetin). The predominant isoflavones accumulated in both callus and cell suspension cultures were puerarin, daidzin, malonyl daidzin, and malonyl genistein. Greater levels of these isoflavones were found in root-derived callus (20.68 mg/g total isoflavones) as compared to leaf-derived callus (3.11 mg/g), and suspension cultures accumulated greater amounts of isoflavones per g cells than the callus. Root-derived suspension cultures accumulated 32.88 mg/g total isoflavones, and leaf-derived suspension cultures had 15.94 mg/g total isoflavones. Untransformed root cultures developed from roots of in-vitro *P. lobata* plantlets, grown in liquid MS medium (supplemented with 1 mg/ml NAA), produced significantly higher amounts of puerarin and daidzin than the root-derived suspension cultures (14.25 mg/g puerarin in root cultures vs. 1.57 mg/g puerarin in root-derived suspension cultures; 8.17 mg/g vs. 4.90 mg/g for daidzin), but had similar levels of total isoflavones (29.91 mg/g total isoflavones in root cultures). Analysis of root cultures by LC-MS and MS/MS revealed the presence of several novel isoflavone C-glycosides, which may be intermediates in the pathway of isoflavone synthesis. Root-derived *P. lobata* cell suspension cultures were elicited at day 5 after subculture for 7d with the abiotic elicitor KCN (0.1 micromolar, 1.0 micromolar) to test its effects on isoflavone production, however, elicitation with KCN resulted in no difference in isoflavone production compared to the control.

P-2021

Quantitation of In Vitro Red Clover Isoflavones. NANCY ENGELMANN¹, Randy Rogers¹, Kenneth Jones², Jeevan Prasain², Stephen Barnes², and Mary Ann Lila¹. ¹Department of Natural Resources and Environmental Sciences, 1024 Plant Sciences Laboratory, University of Illinois, Urbana, IL 61801 and ²Department of Pharmacology and Toxicology, Room #456 McCallum Building, University of Alabama at Birmingham, AL 35294. Email: nengelma@uiuc.edu

Cell cultures from red clover (*Trifolium pratense*) were investigated as a source for biologically active isoflavones, which have potential benefits for bone density, cardiovascular health, and cancer prevention. In nature, variable levels of isoflavones are produced in certain tissues and in response to growing conditions. Red clover seeds were surface sterilized and 21 d old *in vitro* germinated seedlings were used as a source of vegetative explants. Leaf, root, and petiole explants were used to initiate callus on Gamborg B5 medium with 2 mg/L NAA, 2.25-mg/L 2,4-D, and 2.12-mg/L kinetin. Callus cultures were maintained by subculturing at 4 wk intervals and then used to induce suspension cultures that were subcultured at 2 wk intervals. Both callus and suspension cultures were grown in darkness or light (100 mmol/m²/s). Cells were subsequently harvested, extracted with methanol, and analyzed for isoflavone content using LC-MS and MS/MS. Leaf tissue explants were the most prolific source of callus cells, whereas solution cultures produced the most biomass when root explants were the donor tissue for callus. The predominant isoflavones recovered were formononetin, biochanin A, daidzein, and genistein. On average, 10-fold higher yields of formononetin [0.67 mg/g fresh mass (FM)] and biochanin A (0.13 mg/g FM) were found in petiole-derived solution cultures maintained in darkness.

P-2022

In Vitro Production of Secondary Metabolites of Potential Clinical Value by Embryogenic Cultures of Nutmeg. R. INDIRA IYER, G. Jayaraman, and A. Ramesh. Department of Genetics, Dr. ALMPGIBMS, University of Madras (Taramani campus), Chennai-600113, INDIA. Email: riier@yahoo.co.in

Myristica fragrans Houtt., a tropical tree species is the source of the high value spices nutmeg and mace which yield several aromatic substances of medicinal and commercial value. Nutmeg is a dioecious tree with recalcitrant seeds and a long generation time. There is a need to explore the potential for *in vitro* production of secondary metabolites by the cultured tissues of nutmeg to address market needs and to devise new health-care options. Embryogenic cultures established from zygotic embryos of nutmeg in MS media with activated charcoal and growth regulators were analysed by headspace GC-MS. In addition to α -pinene, sabinene and β -pinene already reported in this species, a number of novel secondary metabolites of potential therapeutic value including squalene and derivatives of myristicin and safrole were detected in the tissues cultured *in vitro*. Presence of phenolics was detected in the ethanolic extract of the spent medium. The ethanolic extract exhibited anti-bacterial activity against *Salmonella typhi* and *Staphylococcus aureus*.

P-2023

Culture Parameters Influencing the Production of Gossypol and Related Compounds in Cotton Hairy Roots. S. C. MOSS¹, M. K. Dowd¹, S. M. Pelitire², and B. A. Triplett¹. ¹University of New Orleans, New Orleans, LA 70124 and USDA-ARS, Southern Regional Research Center, New Orleans, LA 70124. Email: smoss@src.ars.usda.gov

Conditions for culturing hairy roots from *Rhizobium rhizogenes*-transformed *Gossypium hirsutum* and *Gossypium barbadense* were reported last year. Significant levels of gossypol and related derivatives were produced in culture. Gossypol is a di-sesquiterpene that has antiviral activity against enveloped viruses such as HIV and inhibits the growth of numerous parasitic organisms, fungi, microbes, and insects. Two on-going clinical trials are testing the efficacy of gossypol as a human chemotherapeutic agent. In this study, several culture parameters were investigated to define the optimal biomass and gossypol production conditions for cotton hairy root cultures. Cultures were grown on Murashige-Skoog or Gamborg's B5 media, pH 5.8 with 0, 10, 20, 30 g/L glucose or 0, 10, 20, 30 g/L sucrose. Gossypol levels were not greatly influenced by sugar type but were significantly affected by media composition. Cultures grown on Gamborg's B5 media produced higher levels of gossypol and related compounds than cultures grown on Murashige-Skoog media. We are testing whether this difference is due to total nitrogen content or nitrogen form. Temperature effects (25°, 28°, 31°, 34° C) on hairy root growth and gossypol production were also investigated. Gossypol productivity was greatest at 31° C and production of methylated forms of gossypol was greatest at 34° C. We will also report on the variability of gossypol production after sub-culturing transformants individually for several months and provide gossypol production data for 26 independently transformed lines. Establishing optimal growing conditions and gossypol levels produced by cotton hairy root cultures benefits our long-term goal of characterizing the gossypol biosynthetic pathway.

P-2024

Genetic Improvement of Traditionally Used Medicinal Plants of India for Superior Varieties with High Value Compounds. Akhilesh Kumar and SHARMILA CHATTOPADHYAY. Cellular Biochemistry, Indian Institute of Chemical Biology, Kolkata, 700032, INDIA. Email: sharmila@iic.res.in

Plants continue to be used worldwide for the treatment of disease and novel drug entities continue to be developed through research. In India still a large number of population are unable to afford pharmaceutical drugs and they continue to use their own system of indigenous medicine that are mainly plant based. In search of DNA damage protective agent traditionally used medicinal plants of India were evaluated. Frontline medicinal herb of high commercial value was found as a valuable biosources of herbal antioxidant in the present study, potential DNA damage protective activity of this edible plant is an additional benefit. Progress in biotechnologies offers an alternative promising way to improve genotype of high value medicinal herbs by gene transfer. Establishment of an efficient regeneration system would make a significant contribution in improving qualitative and quantitative characters of medicinal herbs. To establish an efficient regenerative protocol from leaf discs of *Mentha piperita* in the shortest possible period is a major achievement. The present research could be a valuable tool for an in vitro regeneration and genetic transformation systems not only in *Mentha piperita* but also in other cultivars. Glutathione is a tripeptide thiol and one of the most important non-enzymic antioxidant. It is the most abundant intracellular thiol in plant and other organisms and is involved in the synthesis of proteins, DNA and enzyme cofactors. Glutathione plays a significant role in maintaining the protein SH groups in the reduced state, and in removing toxic peroxides formed in metabolism. A deficiency in GSH enhances cell lyses due to lipid peroxidation. Total glutathione was determined by DTNB assay from the fresh leaves of *Mentha piperita* and noted that fresh leaves contain significant amount of glutathione. With these as the background we designed to develop a potential source of natural antioxidant using recombinant DNA technology. The approach is the intervention at molecular level by PCR cloning of γ -ECS and GS. Chloroplastic over expression of γ -ECS and GS in *M. piperita* will increase the desired product, i.e., GSH.

P-2025

Mapping Fertility Restoration Gene, *Rf4*, for WA-cytoplasmic Male Sterility in Rice (*Oryza sativa*). A. AHMADIKHAH^{1,2}, V. S. Sheveloukha,¹ and G. I. Karlov¹.¹Timiriyaev St., No 45, Dept. of Biotechnology, Moscow Agricultural Academy named after Timiriyaev, 127550, RUSSIA and ²Beheshti St., Gorgan University of Agriculture & Natural Resources, I. R. IRAN. Email: ahmadikhah@narod.ru

Rice is the principal food crop of half of the world's population and also serves as a crop research system to understand yield, hybrid vigor, and disease resistance. Restorer genes are a good example where phenotyping is very time-consuming and requires determination of spikelet sterility in testcross progeny. To map the *Rf4* gene conferring restoration fertility for WA-CMS system, we developed a F₂ population consisted from 230 individuals obtained from crossing a WA-CMS line (Neda A) with a restorer line (Amo11). Mapping population was genetically analyzed to detect recombination points between the *Rf* gene and a series of molecular markers. In the F₂ sterile and fertile bulks four SSR markers (RM304, RM171, RM5841, and RM228) on long arm of chromosome 10 showed a linkage with the *Rf4* gene. *Rf4* was flanked by two markers RM171 and RM304 with distances of 3.0 and 1.8 cm, respectively. The two flanking SSR markers give promise of application in molecular marker-assisted selection (MAS) for restorer lines in WA-CMS system, and also for starting chromosome landing for cloning and isolating of *Rf4* gene in the near future.

P-2026

Effect of Different Parameters on Agrobacterium Mediated Gene Transfer in Cherry Tomato Cultivars. BETSY AMPORFUL and Seema Dhir. Department of Biology, Fort Valley State University, Fort Valley, GA 31030. Email: dhirs0@fvsu.edu

Seeds of Cherry tomato (*Lycopersicon esculentum*) cultivars- Orange Pixie, Little Girl, Christmas Grapes and Sun Gold- were germinated on MS media. Two week old seedlings were used as a source of leaf explants. *Agrobacterium tumefaciens* containing PTC 55 vector was used to genetically the leaf explants. We tested the effect of Pre-culture period (0-3 days), duration of co-cultivation (10-30 mins) and different O.D levels (0.4-1.2) on the transformation efficiency. Results were recorded. Screenable marker Gus was used to record results. Our results indicated that the duration of co-cultivation did not have an effect on the genotypes because all leaves turned blue; the different O.D levels turned all the leaves blue, signifying that any O.D level ranging from 0.3 to 1.2 can be used; and the Pre-culture period of leaves did not make a significant difference in bacteria intake.

P-2027

Production of Additional Allotetraploid Somatic Hybrids Combining Mandarins with Pre-selected + Pummelo as Potential Candidates to Replace Sour Orange Rootstock. J. W. Grosser, G. ANANTHAKRISHNAN, P. Serrano, and M. Calovic. University of Florida, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850. Email: anantha@crec.ifas.ufl.edu

Sour orange rootstock has historically been a premier citrus rootstock throughout the world due to its wide adaptability and superior horticultural performance. However, quick-decline isolates of citrus tristeza virus (CTV) have demolished entire industries on sour orange rootstock in some countries, including Brazil and Venezuela. CTV is presently destroying millions of trees on sour orange rootstock in Florida, and threatens the citrus industries of Texas and Mexico where sour orange is the predominant rootstock. Efforts to replace sour orange rootstock are combining traditional breeding and new biotechnologies including somatic hybridization and transformation. Molecular techniques have confirmed that sour orange is probable hybrid of mandarin and pummelo. A major focus of our program is to pre-select superior mandarin and pummelo parents, followed by somatic hybridization via protoplast fusion. Here we report the regeneration of allotetraploid somatic hybrid plants from 7 new mandarin + pummelo combinations and 3 new sweet orange + pummelo combinations. This is a continuation of our earlier somatic hybridization work to develop an adequate CTV-induced quick-decline resistant replacement for sour orange rootstock. All new somatic hybrids were confirmed by leaf morphology, flow cytometry (for ploidy) and RAPD (for nuclear hybridity). Most of the new somatic hybrids are being propagated by tissue culture or rooted cuttings for further evaluation of horticultural performance in field trials.

P-2028

An Easy and Convenient Method for Maintenance of Embryogenic Cultures of *Vitis vinifera* for Propagation or Transformation Purposes. P. CROCE. Laboratory of Plant Biotechnology, Università dell'Insubria, Dipartimento di Ambiente, Salute e Sicurezza, Via Dunant3, 21100 Varese, ITALY. Email: piobio@iol.it

We have developed an easy and convenient method for the maintenance of embryogenic potential of some Italian grape cultivars of economical relevance. We first tested the susceptibility of different Italian grape cultivars (Trebiano d'Abruzzo, Trebbiano Toscano, Sangiovese, Regina, Bombino Bianco, and Montepulciano) to produce somatic embryos from immature anthers and ovaries. We then tested the morphogenic capacity of the somatic embryos from the different cultivars in order to find a high efficiency method for the maintenance of embryonic cell culture. With the method we found is possible to generate an indefinite number of somatic embryos suitable for propagation or to set the conditions for transformation purposes. This method is based on recurrent cycles of induction of somatic embryogenesis using, as source of cells single somatic embryos. Somatic embryos were able to produce embryogenic callus on a 2,4-D containing medium. The embryogenic callus produced on the induction medium was able to generate somatic embryos on a suitable medium. The embryos formed works as starting material for the following cycle. In any cycle the number of embryos can be increased up to a 20 factor. There's no need of any selection of the material on subcultures. We have been growing our cultures with this method for more than two years with no decrease of embryogenic potential.

P-2029

Regeneration and Cloning of *Stevia* Plant: A Low Caloric Sweetener. SHIREEN DHIR¹, K. Knowles², and S. K. Dhir². ¹Feagin Mill Middle School, Houston County, Warner Robins, GA 31088 and ²Center for Biotechnology, Fort Valley State University, Fort Valley, GA 31030. Email: dhirs0@fvsu.edu

Stevia is a perennial herb is valued for natural source of sweetener production. Conventional propagation method through cutting and poor seed germination does not meet the current demand for plating stock and for larger scale extraction of sweetener from the leaves. Our goal is to devise an economically-feasible cloning technique to produce large numbers of clones of selected high content sweetener genotypes. An efficient regeneration system for large scale propagation of *Stevia rebaudiana* was developed using shoot tip and axillary buds harvested from the actively growing shoots of greenhouse grown plants. Explants were cultured on Murashige and Skoog's (MS) Media with different concentration of Benzyladenine (BA, 0-3.0 mg/L) and Thidiazuran (TDZ, 1.0 mg/L) alone or in combination. The highest average number of shoots (two sub-culture/inoculations) in the after 4-6 weeks was obtained with 3.0 mg/L of BA, or 3 g/L of TDZ. On average 30-50 shoots per explant were observed. The production of shoots were further induced in subculture of the original explant, after the first harvest of shoots (stump), on MS medium with TDZ (3.0 mg/L) using similar condition. Green true shoots with fully developed leaves were observed in almost 80% of initial cultures. Comparing three sugars—glucose, maltose, and sucrose, glucose produced the maximum number shoots. The maximum elongation of shoots was achieved with 3.0 mg/L of BA, also it allowed the production of more shoots with a size suitable for rooting. Roots were induced in 3-5 cm long shoots, transferred to MS medium individually supplemented with IAA or IBA (1-4 mg/L). Plantlets were successfully acclimatized and grown in natural conditions. The shoots rooted easily on half strength MS medium. *In vitro* propagated plants could be transferred to soil with survival rates of more than 95%.

P-2030

Phenolic Compounds Produced in Shoots and Roots of Plantlets and Hairy Roots of *In Vitro* Cultures of *Mentha piperita* L. Ana Patricia P. Guedes¹, Margarida Oliveira², Paulo S. C. Braga¹, Carla A. C. Araújo¹, Paula B. Andrade³, Patrícia C. R. Valentão³, Rosa Seabra³, and MANUEL FERNANDES-FERREIRA¹. ¹Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, PORTUGAL; ²ITQB/IBET, Av. da República (EAN), 2781-901 Oeiras, PORTUGAL; and ³CEQUP/Pharmacognosy Laboratory, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha, 4050 Porto, PORTUGAL. Email: mfferreira@bio.uminho.pt

In vitro cultures of *Mentha piperita* plantlets were obtained and routinely maintained by micropropagation of nodal segments on agar solidified MS medium supplemented or not with one auxin and one cytokinin. Hairy roots were induced from leaves of *in vitro* growing plantlets, using *Agrobacterium rhizogenes* strain A4 in the transformation process. Tentative of transformation using a strain of *Agrobacterium tumefaciens* containing the genes *rolABC* in a binary vector, were unsuccessful. However, the multiplication and maintenance of hairy roots cultures induced with *Agrobacterium rhizogenes*, in medium devoid of growth regulators have been performed over the last two years. The phenolic extracts obtained, with ethanol, separately from the aerial part and roots from *in vitro* plantlets as well as from hairy roots were analysed by HPLC-DAD. The highest levels of phenolic acids were found in non-transformed roots, followed by hairy roots and finally by the aerial part of the plantlets.

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P-2031

Metabolomics in *In Vitro* Cultures of Sage (*Salvia officinalis* L.). Paulo S. C. Braga¹, Paula C. Santos-Gomes¹, Patrícia C. R. Valentão², Rosa M. Seabra², Paula B. Andrade², and MANUEL FERNANDES FERREIRA¹. ¹Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, PORTUGAL and ²CEQUP/Pharmacognosy Laboratory, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha, 4050 Porto, PORTUGAL. Email: mfferreira@bio.uminho.pt

Cells suspensions of sage (*Salvia officinalis* L.) were established in liquid MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN). Good suspensions majority constituted by single cells and microcalli were formed with 0.05 mg/L 2, 4-D and 0.5 mg/L KIN. In these conditions cell viability, determined through the esterase activity on fluorescein diacetate, was higher than 80%. The faster growth rates ranged from 2.2 to 3.5 doubling time days depending on the initial cell density and age of the culture. The phenolics, extracted with acetone and methanol, were analyzed and identified by HPLC-DAD. The lipidic compounds extracted with n-hexane, in Soxhlet apparatus, and essential oil compounds obtained by hydrodistillation of the cells and by n-hexane extraction from the medium, were analyzed and identified by GC and GC-MS. Five phenolic acids, three flavonoids and three phenolic diterpenes were identified. Twelve fatty acids a triterpene hydrocarbon and two sterol compounds were identified in the lipid hexane extract. Over the growth cycle drastic changes (>80%) in the levels of the phenolic acids occurred as well as in the relative levels of the major fatty acids. In the hydrodistillates of the suspended cells, 28 compounds, known as constituents of the essential oil of sage, were identified. Sixteen of these essential oil constituents were found also in the medium. In plants of sage growing in Nature, the essential oils accumulate in the glandular trichomes, majority differentiated on the surface of the leaves. This work shows that sage suspended cells express the synthesis of a lot of the sage essential oil constituents notwithstanding the absence of differentiated glandular trichomes.

P-2032

Evaluation of Regeneration Capacity through Immature Embryo Culture in Soybean and the Identification of Associated RAPD and SSR Markers. Wenbin Li¹, Yingpeng Han¹, Dezhu Yao¹, Shifeng Li¹, Meilu Yang¹, Genlou Sun². ¹Soybean Research Institute, Northeast Agricultural University, Harbin, CHINA 150030 and ²Biology Department, Saint Mary's University, Halifax, Nova Scotia, B3H 3C3 CANADA. Email: wenbinli@neau.edu.cn

The genetic knowledge of regeneration traits based on soybean immature embryo culture and the discovery of molecular markers associated with these traits have great advantages for efficiently developing elite lines with elevated regeneration capacity by increasing selection precision. But less information, in our knowledge, is available for the association of plant regeneration and molecular markers in soybean. In the present report, 95 soybean accessions collected from several countries were evaluated for regeneration efficiency through immature embryo culture using inducible medium containing MS salts + B5 Vitamins + 10 mg/l NAA + 0.5% sucrose + 0.2% Gelrite gellan gum (pH 7.0), and RAPD/SSR markers associated with the regeneration characters was identified. Our result showed that more than 70% accessions possessed regeneration ability in this culture system. Of 250 SSRs tested, nine SSR markers were detected to be relevant to percent regeneration for somatic embryos and seven SSR markers were associated with the average numbers of somatic embryos per explant. Of 1100 RAPDs tested, Six RAPD markers were detected to be relevant to percent regeneration for somatic embryos and four RAPD markers were associated with the average numbers of somatic embryos per explant. Significant correlations ($P < 0.01$) existed between percent regeneration and marker index and between average numbers of somatic embryos per explant and marker index. The information provided here might benefit to determine the elite lines of soybean with moderate regeneration response related to immature embryo culture pathway and benefit for the selection of high regenerable soybeans using marker-assisted selection.

P-2033

A Normalized Transient Expression System To Study Transgene Expression. H.-M. MA¹, R. Griesbach², and M. Pooler¹. USDA/ARS U.S. National Arboretum, Floral and Nursery Plants Research Unit, 13501 New York Ave., NE, Washington, DC 20002 and ²10300 Baltimore Ave., Beltsville, MD 20705. Email: mah@ars.usda.gov

Transient expression has a wide range of applications in molecular biology. It is often carried out by direct DNA transfer rather than *Agrobacterium*. The expression of genes on the plasmid DNA can be detected very soon. One can easily test a large number of putative important transgenes in a relatively short time period before committing to the most promising candidate gene for stable transformation. However, variables inherent in the tissue culture and bombardment processes make it difficult to compare data from independent transformation events. The goal of this work was to develop a normalized transient expression system by introducing two control reporter constructs along with a construct of interest. The reporter constructs contained either *ZEAmayMYC*; or *ZEAmayMYB*; (*Zea mays* anthocyanin regulatory genes) under the control of the CaMV 35S promoter. These two genes are capable of activating anthocyanin biosynthesis in white *Phalaenopsis amabilis* flowers. The resulting anthocyanin production was used to standardize transient expression. The test constructs included five plasmids, each containing a different promoter fused to a bifunctional *GFP:GUS* reporter gene. Using the petals of white *Phalaenopsis amabilis* flowers, the promoter activity was evaluated by transient co-expression of three constructs, one test construct and two control constructs.

P-2034

Determination of Tomatoes, Flavor Producing Biochemical Pathways. ISAAC MICKENS¹, Tiffany Lester¹, and Harry Klee². ¹Center for Biotechnology, Fort Valley State University, Fort Valley, GA 31030 and ²Department of Horticultural Science, University of Florida, Gainesville, FL 32611. Email: dhirs0@fvsu.edu

For the past three years, at the University of Florida extensive research has been done to improve the flavor and map the biochemical pathway, which gives rise to the volatiles essential to a tomatoes flavor. Flavor is a complex interaction among **Sugars** (*glucose, fructose*), **Acids** (*citrate, malate, ascorbate*), and **Volatiles** (*derived from carotenoids*). All three of which play a very important role in how the human tongue detects and interprets what we call flavor. Sterilized seeds of tomatoes are grown on Murashige and Skoog's medium (1/2 MSO2%); two days in the dark and four days in the light. Later the cotyledons are removed from the seedlings and transferred to C media (NAA & BAP) and co-cultivated with *Agrobacterium* (Yep liquid culture, 1 loop glycerol stock in 100ml w/antibiotics *Kan/Spec*) for 30 min. The *Agrobacterium* is the plasmid used to transfer our suspected flavor producing gene of interest. After 30 min. the *agrobacterium* is then drained, and the Cotyledons are left to grow in direct florescent light at 27° C for three days. The transgenic plants are transferred five more times to various types of medium, before reaching soil (agro mix, IBA powder). PCR test is later performed to verify successful transgenic plants by checking for kanamycin in the plants DNA.

P-2035

Effect of Chlorate6, a Moco Biosynthetic Gene, on Arabidopsis Root Growth Responses to Abiotic Stress and Exogenous Hormones. RHEA MOORE¹ and Mark Settles². ¹Fort Valley State University, Fort Valley, GA 31030 and ²Department of Horticultural Science, University of Florida, Gainesville, FL 32611. Email: dhirs0@fvsu.edu

The molybdenum cofactor (Moco) is utilized by enzymes important for the synthesis or metabolism of abscisic acid (ABA), auxin, and nitrate. The chlorate6 (chl6) mutant in Arabidopsis has a point mutation in the Cnx1 gene, which is required for Moco biosynthesis. The chl6 mutation causes reduced nitrate reductase activity, which is a Moco-dependent enzyme. We hypothesized that chl6 plants would have reduced ABA and auxin synthesis causing increased sensitivity to ABA, auxin, and abiotic stresses. This hypothesis was tested with quantitative root growth assays in the presence of exogenous mannitol, salt, ABA, and auxin. In comparison to the wild-type, *Lansberg erecta*, chl6 mutants showed slight hypersensitivity when grown on low levels of ABA or auxin, consistent with reduced hormone levels. Surprisingly, the chl6 mutants showed no differences in response to abiotic stresses suggesting that chl6 mutants have near normal induction of ABA synthesis. These results suggest that the chl6 point mutation has differential effects on Moco-dependent enzymes, because the chl6 mutation was originally selected for a nearly complete loss of nitrate reductase activity.

P-2036

Plant Regeneration from Callus Cultures Derived from Mature Zygotic Embryos in White Pine (*Pinus strobes* L.). W. Tang and R. J. NEWTON. Department of Biology, Howell Science Complex, East Carolina University, Greenville, NC 27858-4353. Email: Tangw@mail.ecu.edu

Plant regeneration via adventitious shoot organogenesis from callus cultures initiated from mature embryos in white pine (*Pins strobes* L.) has been obtained in this study. Callus cultures were induced from mature embryos cultured on PS medium supplemented with 2,4-Dichlorophenoxyacetic acid (2, 4-D), α -Naphthaleneacetic acid (NAA), or Indole-3-acetic acid (IAA). Adventitious shoot regeneration from callus cultures was induced on medium containing 2 μ M (IBA) and 3-12 μ M N₆-benzylaminopurine (BA), thidiazuron (TDZ), or 6-(γ , γ -dimethylallylamino) purine (2iP). Sucrose is the most suitable sugar for adventitious shoot formation in white pine. The highest frequency (71.7%) of shoot formation was obtained after callus cultures were treated at 4° C for six weeks. The frequency of adventitious shoot formation increased when 0.1 mM putrescine was added to the basal media supplemented with 6 μ M TDZ and 2 μ M IBA. Putrescine improved adventitious shoot organogenesis by decreasing lipid peroxidation. These findings provide useful information on adventitious shoot organogenesis and may be valuable for genetic transformation in white pine.

P-2037

Differential Expression of the "Super-promoter" in Leaves and Hairy Roots of Tobacco. LUIS H. NOPO-OLAZABAL, Bonnie J. Woffenden, Deborah G. Reed, W. Scott Buswell, Chenming Zhang, and Fabricio Medina-Bolivar. ¹Department of Plant Pathology, Physiology and Weed Science and ²Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061. Email: fmb2@vt.edu

The identification of promoter systems for high level constitutive or inducible expression of transgenes is desirable in plant biotechnology. To this end, we have characterized a chimaeric promoter composed of the "super-promoter" (Super P; Ni *et al.*, Plant J., 7: 661-676, 1995) and the translational enhancer from tobacco etch virus (TEV). Analysis of cis-regulatory elements in the Super P sequence showed stress responsive and root meristem-specific elements. We developed transgenic tobacco plants and hairy roots with the Super P:TEV promoter and a modified green fluorescence protein (mGFP5) as a reporter gene. To exploit the utility of the hairy root cultures as a secretion-based expression system, we fused the signal peptide of patatin with GFP to direct its secretion into the medium. Southern blot analyses showed the presence of 1 to 5 copies of the transgene, and Northern blotting verified the expression of GFP in all the transgenic plants. Interestingly, GFP mRNA levels were approximately 3-fold higher in younger than mature leaves. The highest levels of expression were observed in actively growing hairy roots with GFP mRNA levels 2-fold higher than in younger leaves. Immunoblotting with anti-GFP antibodies showed a doublet band of 27 and 28 kDa in all leaf and hairy root extracts, whereas secreted GFP was present as a single protein band of 27 kDa. Plant lines p2 and p8 with 310 and 520 ng GFP per mg of leaf total soluble protein (TSP), respectively, were identified as the best expressers. Hairy roots (14-day old) developed from plant line p2 showed 581 ng of GFP per mg of root TSP and secreted 40 ng of GFP per ml of culture. Inducibility of the promoter was studied with mature leaves from line p2. A 2.1-fold increase in mRNA levels was found immediately after wounding, with a corresponding increase in GFP protein 24 hours after wounding. Studies of wound induction of hairy roots are underway. Results on inducibility of Super P promoter in hairy roots will facilitate its use for the production of recombinant proteins under controlled environmental conditions.

P-2038

Transgenic 'Hamlin' Sweet Orange Plants Containing a Rice Xa21 cDNA Gene Obtained by Protoplast/GFP Transformation. A. A. OMAR and J. W. GROSSER. University of Florida, Citrus Research and Education Center, Lake Alfred, FL 33850. Email: omar@crec.ifas.ufl.edu

'Hamlin' orange (*Citrus sinensis* (L.) Osbeck) is one of the leading commercial cultivars in Florida because of its high yield potential and early maturity. 'Hamlin' also has a high regeneration capacity from protoplasts and is often used in transformation experiments. Citrus canker disease caused by the bacterial pathogen *Xanthomonas axonopodis* pv. *citri* is becoming a worldwide problem. Xa21 gene is a member of the Xa21 gene family of rice which provides broad spectrum *Xanthomonas* resistance in rice. Since the citrus canker pathogen is in the same genus, this gene may have potential to function against canker in citrus. The wild-type Xa21 gene contains an intron, and there is some question as to whether dicot plants can process genes containing monocot introns (the cDNA is intron free). The development of canker resistant sweet orange has become an important research objective. Plasmid DNA (pAO3), encoding the non-destructive selectable marker EGFP (Enhanced Green Fluorescent Protein) gene and the cDNA of the Xa21 gene, was transformed into 'Hamlin' orange protoplasts using polyethylene glycol. Following protoplast culture in liquid medium and transfer to solid medium, transformed colonies were microscopically selected via expression of GFP, physically separated from non-transformed tissue, and cultured on somatic embryogenesis induction medium. More than 75 transgenic embryoids were recovered. They are growing rapidly and should develop into transgenic plantlets soon. So far, 19 transgenic plantlets were developed from three independent transgenic events. PCR analysis revealed the presence of the cDNA of the Xa21 and the GFP genes in the transgenic plantlets. The recovery of replicate transgenic plants for a canker challenge assay will be expedited by *in vitro* grafting and shoot-tip grafting in the greenhouse. Southern blot analysis to confirm the presence of the Xa21 cDNA gene and to determine copy number will also be performed on the transgenic plants once we have enough material to extract genomic DNA. Transgenic plants will be assayed for canker resistance using standard methods.

P-2039

Production of *Acidothermus cellulolyticus* Endoglucanase (E1) Enzyme in Transgenic Rice. HESHAM ORABY, Rashid Ahmad, Callista Ransom, and Mariam Sticklen. Department of Crop and Soil Sciences, Plant and Soil Science Building, Michigan State University, East Lansing, MI 48823. Email: orabyhes@msu.edu

Rice is an important crop in the USA as well as in developing nations. While seed is the useful portion of this food crop, its biomass has limited use. In fact, in California, excessive piles of rice straw have accumulated because its burning was phased down by the Straw Burning Reduction Act enacted in 1991. In addition to its seeds, rice biomass could be used as a viable bio-based energy crop with potential benefits relative to energy availability, environmental clean-up, and economic security. Commercial production of ethanol from plant biomass sources employs enzymatic hydrolysis of cellulose to fermentable sugars. At present, these enzymes are produced in fermentation tanks and sold at extremely high costs. Transgenic plants that can produce their own hydrolysis enzymes can become an economically viable alternative to microbial fermentation systems for production of industrial enzymes and other molecules, and help recycle the contents of excessive agricultural wastes such as rice straw. The aim of this study was to develop transgenic rice plants that produce a high amount of active microbial (*Acidothermus cellulolyticus*) endoglucanase (E1). The *Agrobacterium* strain LBA4404 was used to transform mature seed derived calli of the rice cv. Taipei 309 to transfer a plasmid construct containing the *e1* gene designed for apoplast-targeting of the E1 enzyme, β -glucuronidase (*uidA*; *gus*) and the *bar* herbicide resistance selectable marker genes. Secretion of the enzyme had neither deleterious effects on plant growth rate, nor plants exhibited abnormal phenotypes correlating with expression of the enzyme in transgenic plants. Molecular (PCR and Northern blot analyses) and histochemical (GUS) studies confirmed the presence and expression of all three transgenes in five randomly selected glufosinate ammonium resistant lines. The amount of heterologous E1 enzyme in transgenic rice plants determined by the MUCase activity assay accounted for up to 3.2 % of the plant total soluble proteins. Immunofluorescence Confocal microscopy of transgenic leaves is in progress to show the localization/accumulation of E1 in transgenic plants. With collaboration of a chemical engineering team, work is also in progress to see whether rice-produced E1 would lose activity during Ammonia Fiber Explosion (AFEX) pretreatment (i.e., considered the mildest pretreatment). If so, we plan to produce transgenic plant Leaf Soluble Protein Concentrate (LSPC) including the E1, and add the LSPC to lignocellulosic matter after AFEX pretreatment for enzymatic hydrolysis of pretreated lignocellulosic matter.

P-2040

Clonal Root Technology (CRT) for Target Protein Production. M. V. SKARJINSKAIA, J. R. Karl, and V. M. Yusibov. Fraunhofer USA, Center for Molecular Biotechnology, 9 Innovation Way, Suite 200, Newark, DE 19711. Email: vyusibov@fraunhofer-cmb.org

Hairy roots have been widely used for the production of plant metabolites, and recombinant proteins. Here we describe a novel way of producing target proteins in hairy roots that are generated from single plant cells infected with a plant virus. The genes encoding GFP or human growth hormone (hGH) were engineered into a TMV-based expression vector and used to infect *Nicotiana benthamiana* leaves. Leaves infected with each construct were used to obtain hairy roots by *Agrobacterium rhizogenes*-mediated transformation. Hairy roots obtained from these infected leaves were screened for the presence of GFP or hGH by appropriate methods. GFP-expressing roots were selected under UV illumination and the presence of protein confirmed by Western blot analysis. Roots obtained from leaves infected with hGH-producing vector, were selected randomly and examined by Western blot analysis. Out of 100 individual root lines examined for presence of hGH 86 were positive. The highest-expressing roots were selected and clonal root lines were established. Clonal root lines are being examined monthly by UV illumination and/or by Western blot analysis to confirm the presence of GFP or hGH to evaluate stability of expression. We are currently growing clonal root lines in sterile flasks on a rotary shaker. In 30-45 days the root biomass increases 100 fold. The level of hGH expression in roots is comparable to that in *N. benthamiana* plants infected with the hGH-expressing viral vector.

P-2041

Transgenic Christmas Trees Regenerated from *Agrobacterium tumefaciens*-mediated Transformation of Zygotic Embryos and T-DNA Junction Analysis in Transgenic Plants. W. TANG and R. J. Newton. Department of Biology, Howell Science Complex, East Carolina University, Greenville, NC 27858-4353. Email: Tangw@mail.ecu.edu

Mature zygotic embryos of recalcitrant Christmas tree species Fraser fir [*Abies fraseri* (Pursh) Poir], Nordmann fir (*Abies Nordmanniana* L.k.), and Virginia pine (*Pinus virginiana* Mill.) were used as explants for *Agrobacterium tumefaciens* strain GB3850-mediated transformation using *gfp* (green fluorescent protein) gene as a reporter. Factors including media used for inoculation and co-cultivation, concentrations of acetosyringone, and types of antibiotics in tissue culture media have been evaluated. A high transformation frequency was obtained on TE medium containing 50 μ M acetosyringone and using 500 mg/l timentin to eliminate bacteria. Transient gene expression was observed in all three Christmas tree species, but transgenic plants were only produced from Virginia pine. Stable integration of transgenes in the plant genome of Virginia pine was confirmed by polymerase chain reaction (PCR), Southern and Northern blot analyses. These results demonstrate that a stable transformation system has been established in a Virginia pine and this system would provide an opportunity to transfer economically important genes into Christmas tree species.

P-2042

Analysis and Use of the Tobacco eIF4A-10 Promoter Elements for Transgene Expression. L. TIAN¹, K. Wu², C. Hannam¹, M. Latoszek-Green¹, S. Sibbald¹, M. Hu³, D. C. W. Brown¹, and B. Miki³. ¹Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford St., London, Ontario, CANADA N5V 4T3; ²Department of Biology, West Virginia University, PO Box 6057, Morgantown, WV 26506-6057; and ³Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Ontario, CANADA K1A 0C6. Email: tianl@agr.gc.ca

The eIF4A gene family codes for proteins which unwind secondary structures of mRNA during translational initiation. The tobacco eIF4A-10 promoter is one of a few examples of constitutive promoters found in plants. Research was conducted to identify the proximal promoter elements and to evaluate the potential application of the promoter for regulating transgene expression in a range of crop plants. A large intron (892 bp) in the leader sequence was found to be dispensable for constitutive promoter activity and did not contribute to the overall performance of the promoter. Deletion analysis showed that the upstream region between -151 bp to -73 bp relative to the transcriptional start site was essential for the high level of expression and the constitutive activity. The data indicated that the elements in this region may coordinate and compensate each other for the high levels of promoter expression. The downstream leader sequence also contained a strong quantitative enhancer element that was essential for the full activity of the eIF4A-10 promoter. The eIF-4A10 promoter was found to be active in a wide range of plant species and tissues indicating that it will be useful for the constitutive expression of transgenes in plants.

P-2043

Electroporation Mediated Gene Transfer in *Stevia rebaudiana* Protoplasts. MAHOGANY WALKER and S. K. Dhir. Center for Biotechnology, Fort Valley State University, Fort Valley, GA 31030. Email: dhirs0@fvsu.edu

Protoplasts were isolated from *Stevia rebaudiana* leaves, stems, and petioles of proliferating *in vitro* cultures. Various concentrations of Cellulose Onozuka R-10, Macerozyme R-10, and Driselase were tested. Leaves were incubated in a mixture solution of the three enzymes on a gyratory shaker at 50 rpm under darkness for 4-12 hours. Purified protoplast were cultured on KP8 medium supplemented with 2, 4-D, and BAP as liquid, agar, or hanging drop culture. Different responses were obtained in terms of the efficiency for the first cell division colony and micro callus formation. After 30 days, the liquid cultures produced large quantities of micro colonies. On the contrary only a few colonies were formed in the hanging drop and agar cultures. In order to develop a high efficiency and reproducible transformation protocol, we assessed the biological and physical parameters affecting electroporation of protoplast. Energy input, as combinations of electric field strengths discharged by different capacitors, electroporation buffer, number of pulse and DNA form were evaluated. Transformation efficiency was assayed *in vivo* as transient reporter gene expression, using the GFP-coding gene *mgfp5* driven by a CaMV 35S constitutive promoter. Energy input and electric field strength had a critical influence on transgene expression with higher transformation levels being achieved with 200 V/cm-1 discharged by 900 and 25 μ F capacitors. The optimal condition for the transient expression of GFP gene was obtained when protoplast were electroporated in CPW solution 13 M mannitol, 5.0 mM MES and with EPR buffers at 1 electric pulses of 200/Vcm. The transient expression of GFP gene increased when amount of DNA was increased from 15-25 μ g/ml. Comparing number of pulse, protoplast electroporated at 200 V/cm with 1 pulse yielded high GFP expressing cells (3,600-4,000/ml). This system could be used to rapidly test the efficiency of the promoters and the constructed genes for plant transformation in *Stevia*.

P-2044

The 3' Untranslated Region of the Soybean *GmglN(beta)₁* Gene Controls Gene Expression at the Post-Transcriptional and Translational Level. CAROL POTENZA, Jose Ortega, Fernando Solorzano, and Champa Sengupta-Gopalan. Department of Agronomy & Horticulture, New Mexico State University, Las Cruces, NM 88003. Email: cpotenza@nmsu.edu.

Glutamine synthetase (GS) catalyzes the synthesis of glutamine through the condensation of ammonia and glutamate. It also represents a link between carbon (C) and nitrogen (N) metabolism. Since GS has a key role in plant nitrogen metabolism, we introduced the soybean *GmglN(beta)₁* gene as a 35S-GS₁ construct into alfalfa in order to overexpress the GS₁. Our results show that in the transgenic alfalfa the GS₁ is controlled at the level of transcript stability and translatability of the mRNA. Nitrogen availability has an effect on the accumulation of the GS₁ transcript corresponding to the transgene, suggesting that a product of nitrogen assimilation has a role in the turnover of the GS₁ transcript. However, nitrogen does not appear to play a role in translation of the *GmglN(beta)₁* transcript. Our results also indicate that regulation of the *GmglN(beta)₁* at the transcript stability and translatability levels is mediated by the 3' untranslated (UTR) region. Removal of the 3' UTR from the soybean GS₁ transgene results in high accumulation of the GS₁ transcript and *GmglN(beta)₁* protein product. Because we received the same results in both alfalfa and tobacco expressing the two transgenes (+/-3'UTRs), we postulate that the mechanism of 3'UTR regulation might be universal. To examine the role of the GS₁ 3'UTR in the stability and translatability of the messenger and the mechanisms involved in these processes, we have introduced this regulatory region behind the GUS reporter gene driven by the CaMV 35S promoter. The GUS: *GmglN(beta)₁* 3'UTR transgene is expressed at much lower levels than the control GUS:NOS transgene. Because we have not found conditions that allows for increased levels of either the *GmglN(beta)₁*+3'UTR transgene or protein product in transgenic plants, we are testing different metabolite conditions on the expression of the *GmglN(beta)₁*+3'UTR in tobacco to help shed light on the potential C and N regulation of this gene.

P-2045

The Beta-zein is Not Enough to Stabilize Delta-zein in Transgenic Tissues to High Enough Levels. NINA KLYPINA, Suman Bagga, Carol Potenza, Dennis Sutton, Soumitra Ghoshroy, Stephen F. Hanson, and Champa Sengupta-Gopalan. New Mexico State University, Las Cruces, NM 88003. Email: sbagga@nmsu.edu

Alfalfa is the major forage crop worldwide. However, alfalfa is deficient in methionine (Met), which producers must provide to ruminant animals as high protein supplements for adequate production and output. Efforts using conventional breeding to increase the S-amino acid content of alfalfa have met with limited success and thus the only viable approach to increase Met content in alfalfa is by genetic engineering. The gene for delta-zein, a seed protein from corn, would qualify as an ideal candidate for engineering since the protein has 27% Met and is rumen stable. However, it does not accumulate to high enough levels in transgenic plants to make a difference at the nutritional level. Zeins, the major seed storage proteins of maize, are synthesized on the rough endoplasmic reticulum (ER) and deposited in ER-derived protein bodies where beta- and gamma-zeins have been shown to play a major role in protein body biogenesis. Co-expression of delta-zein and beta-zein genes in transgenic plants showed that delta-zein was co-localized in beta-zein containing protein bodies. The level of delta-zein was five fold higher in delta-/beta-zein plants than in delta-zein plants alone, thereby, suggesting that delta-zein interacts with beta-zein and that the interaction has a stabilizing effect on delta-zein. The focus of this study is to determine if delta-zein can be increased to sufficient levels by increasing the level of the beta-zein. We have engineered beta- and delta-zein genes driven by two different constitutive promoters (CaMV and CsVMV) in the same gene construct. A second construct was made by promoter swapping in order to change the beta-zein to delta-zein ratio. We have used these constructs for *Agrobacterium*-mediated stable transformation and for agroinfiltration of tobacco to analyze the effect of increasing beta-zein levels on the accumulation of delta-zein. Our initial results suggest that delta-zein accumulation cannot be increased to the sufficient levels by just increasing the beta-zein.

P-2046

Use of the Green Fluorescent Protein (GFP) Gene in *Prunus incisa* 'February Pink' as a Selectable Marker. E. J. CHEONG and M. R. Pooler. USDA/ARS, U.S. National Arboretum, Floral and Nursery Plants Research Unit, 3501 New York Ave., NE, Washington, DC 20002. Email: cheonge@ars.usda.gov poolerm@ars.usda.gov

The flowering cherry (*Prunus* species) is a popular ornamental plant in the United States and other countries. The shrub breeding program at the U.S. National Arboretum involves development of new cultivars of ornamental *Prunus* using both traditional breeding methodologies and biotechnology. Due to quarantine restrictions, it has become increasingly difficult to bring new *Prunus* germplasm into the U.S. for breeding; hence, genetic engineering may be one of the few options to introduce genes for resistance to microbial and insect pests into *Prunus*. We have developed an in-vitro regeneration system for one flowering cherry cultivar, *P. incisa* 'February Pink,' through somatic embryogenesis from in-vitro tissues, and transformed this tissue using the Green Fluorescent Protein (GFP) as a selectable marker. The GFP/NPTII fusion gene construct was introduced into somatic embryos derived from root or leaf tissue by *Agrobacterium tumefaciens*. Transformed cells expressing the GFP gene were isolated using a stereo microscope set up for GFP detection. Embryogenic callus and somatic embryos were obtained from GFP-expressing callus cultivated on MS medium containing 10 μ M 2,4-D plus 50 mg/l kanamycin. These embryos were then germinated on MS medium containing 1 μ M BA and 2 μ M GA₃. Transformed plantlets were propagated by shoot cultures. GFP expression in transgenic plantlets was difficult to detect due to the presence of chlorophyll, but callus induced from transgenic leaves expressed the green fluorescent protein. The presence of the GFP/NPTII fusion gene was also detected by PCR analysis using primers inside this region to amplify a 600-bp PCR product. The use of the GFP gene as a selectable marker was successful. This system will be useful in future work to introduce genes for pathogen resistance and ornamental traits into flowering cherry germplasm.

P-2047

Expression of Gal/GalNAc Lectin of *Entamoeba histolytica* in Transgenic Chloroplasts to Develop a Vaccine for Amebiasis. S. CHEBOLU, B. Mann, and Henry Daniell. ¹Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science, Bldg. #20, Room 336, Orlando, FL 32816-2364 and ²Depts. of Internal Medicine & Microbiology, University of Virginia Health System, Charlottesville, VA 22908-1340. Email: daniell@mail.ucf.edu

Amebiasis is caused by *Entamoeba histolytica* and ranks only second to malaria as a protozoan cause of death. WHO estimates that 50 million people are infected annually causing 40-100 thousand deaths. The enteric parasite has no zoonotic reservoirs hence anti-amebic vaccine could completely eradicate the disease. The Gal/GalNAc lectin, an amebic surface antigen mediates attachment to host cells. A carbohydrate recognition domain (CRD) was identified in the heavy subunit and is a potential target to block colonization. Expression of gene fragment encoding the CRD (LecA) in plant cells via chloroplast genetic engineering could result in a low cost vaccine because of high expression levels of vaccine antigens, and elimination of the cold-chain (low temperature, storage & transportation), hospitals and health professionals for their delivery. The Lec A gene was cloned into the tobacco chloroplast vector, pLD-CtV. The pLD-CtV has *trnI* and *trnA* genes that are used as flanking sequences for homologous recombination, the constitutive 16s rRNA promoter to regulate transcription. The *aadA* gene conferring spectinomycin resistance has been used for selection and gene10 regulatory sequence from T7 bacteriophage was used to enhance translation. *Nicotiana tabacum* var. Petit Havana leaves were bombarded using the plasmid DNA, pLD-SC-LecA. The chloroplast integration of LecA was confirmed by PCR and homoplasmy was confirmed by Southern blot analysis. The expression of LecA, a 64-kDa protein in transgenic chloroplasts was confirmed by immunoblot analysis using anti-LecA antibody. Maximum accumulation of the LecA protein (up to 5% of tsp) was observed even in the old leaves, suggesting higher accumulation in mature leaves. The evaluation of the immune response of the vaccine antigen in animal model is underway.

P-2048

Nucleoprotein Gene of Tomato Spotted Wilt Virus as a Protein Fusion for Production and Purification of Recombinant Protein in Plants. C. LA-CORTE¹, D. Lohuis, R. Goldbach, and M. Prins. Laboratory of Virology, Wageningen University, Binnenhaven 11, 6709 PD, Wageningen, THE NETHERLANDS and ¹ EMBRAPA-Recursos Genéticos e Biotecnologia, Brasília, DF, 70.770-900, BRAZIL. Email Cristiano.Lacorte@wur.nl

Upon infection of plant cells, *Tomato spotted wilt virus* (TSWV) forms ribonucleoprotein particles (RNPs), which consist of N protein and viral RNA. These aggregates result from the homopolymerization of the N protein, and are highly stable in plant cells. These properties feature the N protein as a potentially useful plant-based protein fusion system. To evaluate this potential, the N gene was fused to GFP (Green fluorescent protein), either to the N or C terminus, in a binary vector or in a Potato virus X (PVX) vector. *Nicotiana benthamiana* leaves were infiltrated by *Agrobacterium tumefaciens* transient assay (ATTA) or inoculated with the PVX vector. For both expression methods N and N-GFP fusion could be detected by Western blot using antisera against N or GFP. Infiltrated leaves and infected plants expressing N-GFP fusions showed intense fluorescence under UV light, revealing clearly visible aggregates in the cytoplasm. For purification, the standard method used for viral RNPs was tested, consisting of two low speed centrifugation steps and one ultracentrifugation on a sucrose cushion. The purified N-GFP retained its fluorescence and had the expected size, reacting with both N and GFP antisera, in Western blot analysis. These results show that the N protein fusion can be produced in plants and easily and efficiently purified as aggregates, apart of the viral infection context. Furthermore, these purified aggregates will also be tested as an epitope presentation system, possibly avoiding the size and conformation constrains of some of the currently available systems.

P-2049

Expression of a Subunit Vaccine for Pneumonic Plague in Tobacco Hairy Roots. B. J. Woffenden¹, L. H. Nopo-Olazabal¹, D. G. Reed¹, C. Cramer², and F. MEDINA-BOLIVAR¹. ¹Department of Plant Pathology, Physiology and Weed Science, Virginia Tech, Blacksburg, VA 24061 and ²Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR 72401. Email: fmb2@vt.edu

Pneumonic plague is considered among the top 5 potential bioterrorism threats due to the fast spread of aerosolized *Yersinia pestis* and near 100%, rapid lethality of untreated cases. We intend to develop a plant-based subunit vaccine for plague that addresses several key issues: mucosal efficacy, ease of delivery, rapid scalability, safety, and cost. Parenteral administration of a fusion of the plague antigens F1 and V with a non-mucosal adjuvant protected mice against multiple plague strains (Alpar et al., *Adv. Drug Deliv. Rev.* 51:173-201). However, mucosal delivery of antigen should enhance protection against the pneumonic form of disease. Previously we demonstrated that the non-toxic lectin subunit of ricin, ricin B (RTB), is useful as a mucosal carrier/adjuvant. Intranasal administration of RTB:antigen fusion purified from tobacco hairy root cultures was highly effective in stimulating a mucosal immune response in mice (Medina-Bolivar et al., *Vaccine* 21:997-1005). We engineered tobacco to express a hexahistidine-tagged RTB fused to sequence-optimized F1:V (His:RTB:F1:V). Transgenic plants expressed an mRNA of a size consistent with the predicted transcript (2.9 kb). Crude leaf extracts were screened for RTB lectin activity with a functional ELISA based on asialofetuin-binding, and two lines showed levels of binding activity 2-3-fold higher than the control. Hairy roots were generated from these plant lines. Asialofetuin-binding ELISA of crude hairy root extracts confirmed expression of functional RTB fusion protein. Southern blotting of transgenic plant lines and hairy root lines confirmed integration of the transgene into the tobacco genome. Immunoblotting of lactose affinity-purified protein from several hairy root lines using anti-RTB and anti-His-tag antibodies detected a protein of about 90 kDa, consistent with the predicted size of glycosylated His:RTB:F1:V protein. Purified His:RTB:F1:V protein will be used for intranasal immunization in mice, in which stimulation of mucosal immunity will be a first step towards an improved pneumonic plague vaccine.

P-2050

Thidiazuron Induced High Frequency Shoot Induction and Plant Regeneration in *Psoralea corylifolia* - an Endangered Medicinal Plant. M. FAISAL and M. Anis. Plant Tissue Culture Laboratory, Department of Botany, Aligarh Muslim University, Aligarh-202002, INDIA. Email: FaisalM15@yahoo.com

Psoralea corylifolia Linn. (Fabaceae) is an endangered medicinal plant, distributed in tropical and sub-tropical regions of the world. Due to destructive harvesting and lack of proper cultivation, the wild population of this potential medicinal plant has declined very fast. The effect of thidiazuron (TDZ) was studied on in vitro shoot proliferation from nodal explant of *P. corylifolia*. Proliferation of shoots was achieved on Murashige and Skoog (MS) medium supplemented with different concentrations of TDZ (0.5, 1, 2, 3, 4, and 5 μ M). The maximum number (13.6 ± 1.4) of shoots per explant was obtained from nodal segment cultured on 2 μ M TDZ for 4 weeks and this increased to 29.7 ± 2.1 on hormone free MS medium in the 8 weeks. For rooting, the in vitro proliferated and elongated shoots were transferred individually on a root induction medium containing 0.5 μ M indole-3-butyric acid (IBA). Within 4 weeks of transfer, a mean number (4.5 ± 0.5) of roots per shoot were produced. The regenerated plantlets were transferred to 1:1 soil and vermiculite mixture and acclimatized with 80% survival rate. Fully acclimatized plants were planted in the field condition, and performance is being evaluated. The present findings provide the first report on in vitro axillary shoot multiplication from nodal explant of *Psoralea corylifolia* in response to TDZ.

P-2051

Factors Affecting Somatic Embryogenesis in Different Cultivars of Cotton (*Gossypium spp.*). TANVEER KHAN¹, Anil K. Singh², Alok Shukla², and R. C. Pant³. ¹Dept of Biotechnology, Kumaun University, Nainital, INDIA; ²Dept. of Plant Physiology, G.B.P.U.A&T, Pantnagar, Uttaranchal 263145, INDIA; and ³Kumaun University, Nainital, INDIA. Email: tanu_tinkle@yahoo.co.in

Significant progress has been made in developing protocols for the induction of somatic embryos. Biochemical and molecular investigations on SE are critical to understand the mechanisms of polarity development and its differentiation in tissue system. SE involves the effect and interaction of many different crops but there are dismal studies in case of cotton. A prolonged culture period, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets, and a lack of shoot elongation are the problems associated with cotton regeneration. An investigation of this phenomenon is of prime importance. Thus a proper understanding of the factors affecting somatic embryogenesis and recalcitrancy in cotton is a prerequisite for the development of regeneration protocols which may further pave a way for a stable genetic transformation system. Thus we have studied the endogenous levels of proteins, reducing sugars, phenols and free amino acids, which affect somatic embryogenesis in cultures of cotton. Embryogenic cultivars were found to have higher levels of reducing sugars and proteins while free amino acids and phenols were higher in non-embryogenic cultivars.

P-2052

Embryogenic Callus Induction in *Stemona tuberosa* Lour. N. MONTRI^{1,2}, C. Waworsch¹, and B. Kopp¹. ¹Department of Pharmacognosy, University of Vienna, Althanstrasse 14, 1090 Vienna, AUSTRIA, and ²King Mongkut's Institute of Technology Ladkrabang Chumphon Campus, T. Chumko, A.Prateaw, Chumphon, 86160, THAILAND. Email: kmnattay@kmitl.ac.th

The roots of some Stemonaceae species are widely used as insecticide and for medicinal purposes due to the occurrence of various alkaloids. *Stemona tuberosa* Lour. has long been recommended in Chinese and Japanese traditional medicine for the treatment of respiratory diseases and as biological insecticides against human parasites. Utilization of *S. tuberosa* products has been largely as crude drugs and the forests are still the main source. Traditional propagation techniques are not economic in commercial propagation because of low efficiency. Production by *in vitro* culture might be an interesting alternative. The results presented here suggest potential of embryogenic callus induction with further application to produce plantlets via somatic embryogenesis on a large scale. Investigations trying to obtain the best media for callus induction were performed by employing different types and concentrations of cytokinins. The media containing BAP or TDZ were found to promote callus formation. The amount of compact callus was positively correlated with the concentrations of growth regulators. The best results were obtained with 20 μ M TDZ. Friable callus developed from compact callus on MS media supplemented with 5 μ M 2,4-D, 5 μ M BAP, 5 μ M TDZ and 5 μ M IBA. In the third step embryogenic callus was achieved in liquid MS medium supplemented with 3 μ M 2,4-D in the dark with the subsequent formation of somatic embryogenesis.

P-2053

Plant Regeneration via Indirect Somatic Embryogenesis in *Panax notoginseng* (Araliaceae), - a Medicinal Plant. SATISH MANOHAR NALAWADE¹, Chao-Lin Kuo², and Hsin-Sheng Tsay¹. ¹Institute of Biotechnology, Chaoyang University of Technology, Wufong, Taichung 413 TAIWAN and ²Institute of Chinese Pharmaceutical Science, China Medical University, Taichung 404, TAIWAN. Email: nalawadesm@yahoo.com

Panax notoginseng is an important medicinal plant, valued for its two major active constituents ginsenosides (Rb and Rg) used as vitalizing and stimulative agent in traditional medicines. A reproducible method for regeneration of complete plants via somatic embryogenesis in *Panax notoginseng* using callus derived from flower buds has been developed. Primary callus was obtained by culturing explants on Murashige and Skoog's (MS) medium supplemented with 0.5 - 4.0 mg l⁻¹ 2,4-D in darkness for 8 weeks. Embryogenic callus was induced by repeated subcultures on the same medium at 8 weeks of interval for over one year. Embryogenic callus continued to rapidly proliferate on MS medium supplemented with 1.0 mg l⁻¹ 2,4-D incubated in dark and developed normal and abnormal somatic embryos. Normal somatic embryos developed when embryogenic callus was subcultured on half strength MS basal medium devoid of any hormone and incubated in light/dark condition (16:8 hrs). Cotyledonary stage somatic embryos were transferred in MS basal medium supplemented with 0.5 - 8.0 mg l⁻¹ GA₃ for further elongation. Phenotypically normal plants were recovered from converted somatic embryos and *in vitro* flowering after 3 months of culture. Well developed plantlets were transferred to a sand:peat moss mixture and kept in growth chambers for adaptation.

P-2054

Micropropagation of Food Crops in the Micronesian Atolls. DILIP NANDWANI and Arwan Soson. Agriculture Experiment Station, Cooperative Research and Extension, College of the Marshall Islands, PO Box 1258, Majuro, Marshall Islands, MH 96960. Email: dilipnandwani@yahoo.com

The conditions on the atolls of the Marshall Islands impose considerable restrictions on the range of crops, which can tolerate harsh environments. Government is embarking on an economic development strategy, which aims at increased agricultural production in the country. In recent years significant losses have occurred in the field collection of banana, breadfruit, root and tuber crops due to genetic erosion and atoll constraints. Smaller farmers encounter a shortage of planting materials in staple crops. The College of the Marshall Islands through its new laboratory has begun the propagation of selected food crops such as banana, breadfruit, and taro through tissue culture. Several new introductions of FHIA cultivars of banana and taro from regional institutions have shown good tolerance to insect pests and diseases. Tissue culture plants are being bulked up for the field evaluation and distribution to farmers all over the atolls. The first tests plots of FHIA banana cultivars have given promising results in both demonstration plots and in farmers' fields. Selecting and propagating superior individuals have obtained significant gains in the adaptability of many cultivars in the atoll environment. Field trials focus on yield performance, which includes adaptability to local soil and climatic conditions and, more importantly, to the prevailing pests and diseases in the area. Fruit quality acceptance by farmers and local consumers also being monitored and evaluated. These new cultivars still require further testing, validation and possibly adaptation to local conditions, before farmers adopt them.

P-2055

In Vitro Propagation of *Erythronium propullans* V. C. PENCE, J. R. Clark, and S. M. Charl. Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo & Botanical Garden, 3400 Vine Street, Cincinnati, OH 45220. Email: valerie.pence@cincinnati-zoo.org

Erythronium propullans is a spring wildflower, endemic to Minnesota, with only small numbers of individuals in the wild. Flower, bud, and corm tissues of *E. propullans* were surface sterilized in 10% bleach for 5-10 min and rinsed in sterile water. Tissues were cultured on MS medium containing 3% sucrose, 0.22% Phytagel and 100 mg/L active benlate (fungicide) with 2 mg/L 2,4-D \pm 1 mg/L kinetin, 0.5 mg/L BAP + 0.05 mg/L NAA, or 1 mg/L BAP + 1 mg/L kinetin. Cultures were incubated at 26° C. Of 49 cultures initiated, 10% became contaminated, while half of the remaining cultures showed either swelling or the initiation of callus or shoots. Corm proliferation was stimulated, in general, by media with 2,4-D, while shoot proliferation occurred on media containing BAP. Shoots were transferred to media containing IBA or NAA, but rooting did not occur. Cultures were also placed in 4° C for 8 weeks on rooting medium, but this did not stimulate rooting. Culture at 21° C was also not successful. However, 60% of the cultures produced one or more roots when the shoots were incubated at 10° C for one month. This response may reflect the autumn root growth in these species in the wild, although there are reports of anomalous growth in the population from which the source tissue was taken. Further research is underway to determine the viability of these roots in supporting acclimation of plants of *E. propullans* to soil. Research done in collaboration with the Center for Plant Conservation and the Holden Arboretum and supported in part by IMLS grant no. IC-03-04-0062.

P-2056

Rescue of Transformed Avocado Somatic Embryos and Recovery Plants by Micrografting and Ex Vitro Grafting. S. H. T. RAHARJO¹, T. Witjaksono², D. Efendi³, and R. E. Litz¹. ¹Tropical Research and Education Center, University of Florida, Homestead, FL 33031; ²Plant Tissue Culture Laboratory, Bogor Botanic Garden, Bogor 16003, INDONESIA; and ³Department of Agronomy, Bogor Agricultural University, Darmaga, Bogor 16680, INDONESIA. Email: sraharjo@ifas.ufl.edu

Embryogenic cultures of 'Suardia' and 'Hass' avocado cultivars have been transformed with SAMase and antifungal protein (AFP) genes, respectively. However, recovery of complete plantlets and plants directly from the transformed somatic embryos has been difficult, due to the absence of bipolarity of most avocado somatic embryos. Therefore, two procedures for recovering transgenic avocado plants *ex vitro* were developed. Shoots from somatic embryos were micrografted onto *in vitro* seedlings as rootstocks. *In vitro*-derived shoots were also grafted directly onto three-week-old seedlings *ex vitro*. Micrografting success rates of 'Suardia' transformed with SAMase gene and 'Hass' transformed with AFP gene were 87-100% and 69-100%, respectively. *Ex vitro* grafting of the transformed shoots resulted in 51-74% success. Rapid shoot growth (flushing) occurred 4-5 weeks after grafting, and transgenic plants have grown vigorously in the greenhouse.

P-2057

Differentiation and Encapsulation of *Saintpaulia rupicola* Shoots. M. H. RENFROE, E. N. Johnson, and C. J. Elder. Department of Biology, James Madison University, Harrisonburg, VA 22807. Email: renfromh@jmu.edu

In vitro propagation of *Saintpaulia rupicola*, an endangered African violet species, was investigated as a means of germplasm conservation. The effects of different combinations of an auxin and cytokinin on the differentiation of shoots from leaf explants were investigated. Discoloration of the growth medium was common and additions of polyvinylpyrrolidone, activated charcoal, and ascorbic acid were tested for their effects on preventing browning and influencing differentiation. Relatively low concentrations of phytohormones were required to produce shoot proliferation from leaf tissue. Efforts to reduce discoloration of the medium were not found to enhance differentiation of shoots. *De novo* shoots were excised and encapsulated in alginate with or without inorganic nutrients and vitamins. Encapsulated shoots were grown on a basal tissue culture medium, vermiculite, or a potting soil mix and results were compared for growth of shoots into healthy plants with a commercially acceptable morphology. Inclusion of nutrients in the alginate had no significant benefit. Best growth and plant morphology resulted from planting encapsulated shoots on vermiculite. These results may be useful for rapid propagation and outplanting of members of this endangered species.

P-2058

High Frequency Regeneration of Plantlets from Leaf Explants of Commercial Pear (*Pyrus communis* L.) Cultivars 'Onward' and 'Old Home'. Q. SUN^{1,2}, R. E. Davis¹, and Y. Zhao¹. ¹Molecular Plant Pathology Laboratory, ARS, USDA, Beltsville, MD 20705 and ²Shandong Institute of Pomology, Taian, Shandong, P. R. CHINA 271000. Email: zhaoy@ba.ars.usda.gov

Pear (*Pyrus communis* L.) is an important deciduous fruit with high commercial value. However, pear production is often hampered by bacterial, fungal, and viral diseases. As part of our program aimed at improving resistance of pear crops to diseases *via* genetic engineering, we studied the influence of medium composition and growth regulators on adventitious shoot formation from *in vitro* propagated leaf explants of two popular commercial pear cultivars, 'Onward' and 'Old Home'. Among three basal media (NN69, WPM, and C) tested, NN69 appeared to be the most suitable for promoting shoot regeneration. The cytokinin thidiazuron (TDZ) was much more effective than 6-benzylaminopurine (6-BA) in promoting shoot regeneration in both cultivars. The effect of two auxins, indole-3-butyric acid (IBA) and α -naphthalene acetic acid (NAA), was genotype-dependent. Regeneration frequency of 'Onward' reached 90% in NN69 basal medium plus 4 mg l⁻¹ TDZ and 0.7 mg l⁻¹ IBA. Regeneration frequency of 'Old Home' reached 100% when leaf explants were subjected to a two-phase culture regimen. In the initial shoot primordium induction phase, leaf segments were cultured on NN69 basal medium supplemented with 4 mg l⁻¹ TDZ and 0.3 mg l⁻¹ NAA; in the subsequent shoot elongation phase, emerging shoots were cultivated on Murashige-Skoog (MS) medium supplemented with 0.3 mg l⁻¹ BA and 0.1 mg l⁻¹ IBA. A comparative structural analysis revealed that shoot regeneration from 'Onward' leaf explants essentially occurred via direct organogenesis and that shoot regeneration from 'Old Home' leaf explants followed an indirect organogenesis pathway. Shoots regenerated from both cultivars were successfully rooted on 1/4 MS salts supplemented with 0.3 mg l⁻¹ NAA and 2% sucrose. The high efficiency regeneration protocols established in this study make it possible to use commercially important pear cultivars for eventual genetic engineering of disease resistance and for other pear improvement programs.

P-2059

Thidiazuron Induced Somatic Embryogenesis and Organogenesis in Taro. VIRENDRA MOHAN VERMA¹, John J. Cho², Jabukja Aikne¹, and Jina David¹. ¹Cooperative Research and Extension (USDA Land Grant), College of the Marshall Islands, Majuro MH 96960, MARSHALL ISLANDS and ²Department of Plant Pathology, HITAHR-Maui County Research, University of Hawaii, Kula, Maui, HI 96790. Email: vmv_vmv@hotmail.com

The research was carried out to study the effect of thidiazuron (TDZ) on somatic embryogenesis and organogenesis in taro (*Colocasia esculenta* (L.) Schott). Shoot, adjacent to the corm and shoot meristem were used as explants. Murashige and Skoog, 1962 (MS) medium was used throughout the study. Comparative study was undertaken using control in which only TDZ was eliminated from all the treatments. A two-step protocol (initially on medium augmented with 10 μ M 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 μ M TDZ followed by a culture phase with 5 μ M TDZ) resulted in somatic embryogenesis. Globular, heart and torpedo shaped embryos were observed. In organogenesis, cultures were initiated on medium augmented with 2 μ M TDZ and then transferred on medium with 5 μ M TDZ. Shoots were transferred on medium augmented with 5 μ M indole-3-acetic acid (IAA) and 7.5 μ M 6-benzylaminopurine (BAP) for further growth and subsequent multiplication. The number of multiple shoots produced from each explant after two subcultures varied from 40 to 50. MS medium augmented with 2 μ M IAA was used for rooting. Mature plants were recovered from both somatic embryos as well as from induced shoots. Neither somatic embryogenesis nor organogenesis was observed in control. A 12-h photoperiod with a temperature of 28° C day and 24° C night, light intensity of 40 μ mol m⁻² s⁻¹, and 55±5% relative humidity was maintained for multiplication.

P-2060

Effect of Plant Growth Regulators on In Vitro Multiplication and Conservation of Sweetpotato. VIRENDRA MOHAN VERMA, Jabukja Aikne, and Jina David. Cooperative Research and Extension (USDA Land Grant), College of the Marshall Islands, Majuro, MH 96960, MARSHALL ISLANDS. Email: vmv_vmv@hotmail.com

A project aiming towards introduction and field evaluation of micropropagated sweetpotato in the Marshall Islands is being developed with future plans to establish a germplasm bank of sweetpotato. Influence of media composition and hormonal combinations was studied for *in vitro* multiplication and conservation. As a first step, a micropropagation protocol using nodal segments as initial explants was developed. Two media, Gamborg et al. 1968 (B5) and Murashige and Skoog 1962 (MS), were used for the multiplication of sweetpotato, but best results were observed on MS medium. Shoot multiplication was initiated on MS medium augmented with 5 μ M 6-furfurylaminopurine (KIN), 20 μ M gibberellic acid (GA) and 100 mg/L ascorbic acid. The best multiplication and growth rates were observed on MS medium with 1 μ M indole-3-acetic acid (IAA), 10 μ M 6-benzylaminopurine (BAP), 20 μ M GA and 100 mg/L ascorbic acid. In the second step, with the objective of evaluating the best conditions for *in vitro* conservation by slow growth induction, best results were observed on half-strength MS medium supplemented with 1 μ M BAP. MS medium augmented with 2 μ M IAA was used for rooting. A 16-h photoperiod with a temperature of 25° C day and 22° C night, light intensity of 40 μ mol m⁻² s⁻¹, and 55±5% relative humidity was maintained for multiplication.

P-2061

Establishment of a Highly Efficient Protocol for Micropropagation of North American Ginseng. SIJUN ZHOU and Daniel C. W. Brown. Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford St. London, Ontario N5V 4T3, CANADA. Email: zhou@agr.gc.ca

A highly efficient system for plant propagation of North American ginseng (*Panax quinquefolius*) via somatic embryogenesis starting from cotyledon explants has been established with a six stage protocol based on somatic embryo induction, maturation, germination (and tissue recycling), development of plantlets, acclimation into soil, and transplantation to the field. In the somatic embryo induction stage, somatic embryos can be induced from lateral edges and the adaxial surface of cotyledons cultured on growth regulator-free MS (Murashige and Skoog, 1962) medium with high sucrose content and will result in a high frequency response (60%) and number. The usual high frequency of abnormal and fused embryos can be partially controlled by pre-treating the cotyledon explants with 1.0 M sucrose for 48-96 h at 4 °C. The pretreatment also results in an increase of embryogenesis response frequency (75%) and the number of embryos. A maturation stage is important for further development of somatic embryos and subsequent good germination. The optimal maturation medium is: half-strength MS or SH (Schenk and Hildebrandt, 1972) medium containing 3% sucrose and 1% activated charcoal. In the germination stage, gibberellic acid (GA₃) is necessary for breaking quiescence/dormancy of embryos. The optimal level of GA₃ is 10-20 mg l⁻¹. About 54% somatic embryos can produce normal plants. Elongated cotyledons and petioles from abnormal shoots after germination are good explants for somatic embryo production (tissue recycling) and result in high quality and number of somatic embryos. The frequency of embryogenesis on MS medium containing 1.0 mg l⁻¹ 2, 4-dichlorophenoxyacetic acid and 1.0 mg l⁻¹ α -naphthaleneacetic acid is up to 90%, with up to hundreds of embryos on one explant. With this tissue recycling approach, the efficiency of plant propagation of the system is enhanced tremendously. In the plant development stage, activated charcoal is beneficial to the plantlet especially for root development. The elongation medium, 1/2 SH plus 0.5% charcoal, results in 85% of germinated embryos developing into plants with a well-developed root system. Of over six hundred plants that were rooted and transplanted into soil (Promix BX), two hundred plants have been transplanted into the field in May and June of 2004. The growth chamber and field establishment rates, 5 weeks after transplantation, were 95.6% and 93.7%, respectively. Fifty senesced plants (roots with dormant buds) were transplanted into the field in fall of 2004, while the other ones were put into a cooler set at 4° C at the end of 2004 to overwinter. The acclimatized plants grow very well and show the development of a dormant bud and a thickened root structure typical of seed-germinated plants.

P-2062

Transformation of a 95-kb Maize Centromeric DNA Segment Back Into Maize and Analysis of Transgenic Plants. B. H. PHAN¹, C. Smith¹, C. Topp¹, J. Jiang², R. K. Dawe¹, and W. A. Parrott¹. ¹University of Georgia, Athens, GA 30602-6810 and ²University of Wisconsin, Madison, WI 53706. Email: baophan@uga.edu

The ability to introduce high molecular weight DNA into plant chromosomes should accelerate gene identification and may lead to new approaches to studies of genome organization. We previously reported the transfer into rice of BACs containing centromeric DNA inserts from rice and maize. Maize has become an important model for plant centromere research. Maize centromeres are composed of CentC tandem repeat arrays, centromeric retrotransposons (CRs), and a variety of other repeats. One particularly well conserved CR element, CRM, appears to be interspersed thoroughly with CentC. Microprojectile co-bombardment was used to introduce a BAC containing a 95-kb insert of centromeric satellite repeat elements from maize back into maize. This insert has even centromere-specific retrotransposons of maize (CRM). The CRM elements are inserted randomly into either CentC monomers or other retrotransposons. As a control, maize was also transformed with a similarly sized BAC, pAHC25, containing random genomic DNA. Approximately 0.4% of the immature embryos bombarded yielded callus colonies transgenic for the maize BAC centromere and 5.2% for the pAHC25 control. Transformed plants containing the maize centromeric BAC, engineered with pAHC25, had a distinctive altered morphology. The leaves of the transgenic plants are striped, suggestive of the effects of having dicentric chromosome. Data from southern-blot and progeny analyses confirmed the integration and inheritance of the maize BAC centromeric DNA in the T₀, T₁ and T₂ generations, and were inherited as single Mendelian genes.

P-2063

Validation of Real-time PCR Assays for Transgenic Grapevine Analysis. F. Savazzini¹, I. Ciabatti², A. Paternò², U. Marchesi², D. Amadeo², and L. MARTINELLI¹. ¹Istituto Agrario San Michele all'Adige, 38010 San Michele all'Adige (TN), ITALY and ²Istituto Zooprofilattico Sperimentale Lazio e Toscana, Roma, ITALY. Email: Lucia.Martinelli@iasma.it

Grape molecular breeding progressed rapidly both for studying interesting traits and improving quality of vines and products. Thus, European Union introduced a regulation on labelling of genetically modified (GM) grapevines for marketing of propagation material. Scientific grounds and legislative framework entail development, validation and application of valuable analytical methods for GM grape traceability (CE 11/2002). Quantitative Real-Time PCR is the elite tool for accurately detecting foreign genes, estimating transgene copy number and studying exogenous gene expression. As for grapevine, however, this technique is not a routine yet and needs to be improved. Being embarked in projects on grape molecular breeding and GMO detection, we developed a protocol for standardizing and optimizing grape Real-Time PCR, and identified a valuable endogenous gene to be applied as genotype referee and gene copy number standard (NCED2). Then, we tested our analysis on the GM grape plants available in our laboratory (Savazzini *et al.*, *In Vitro Cell. Developm. Biol. Animal*, P-1246). Finally, we developed a practical approach for the validation of our technique, and provided a detailed protocol for the evaluation of detection and quantification limits, linear dynamic range, precision and trueness, specificity and robustness. Our strategy offers consistency and reliability. Research supported by Italian Health Ministry (Project IZS UM 18/2002, national coordinator C. Rondini) and the Autonomous Province of Trento (Project OSSERVA3).

P-2064

Transformation of Sour Cherry Montmorency (*Prunus cerasus* L.) and Sweet Cherry Rootstock Gisela 6 (*Prunus cerasus* × *P. canescens*) Mediated by *Agrobacterium tumefaciens*. Guo-Qing Song and K. C. SINK. Plant Transformation Center, Department of Horticulture, Michigan State University, East Lansing, MI 48824. Email: sink@msu.edu

Sour cherry (*Prunus cerasus* L.) cv. Montmorency and the dwarf, sweet cherry rootstock cv. Gisela 6 (*Prunus cerasus* × *P. canescens*) were both transformed by *Agrobacterium tumefaciens* strain EHA105 carrying the neomycin phosphotransferase gene (nptII) and an intron interrupted β-glucuronidase (GUS) reporter gene (gusA). Whole leaf explants, taken from in vitro stocks, each with four cuts made equidistant and transverse through the midrib, were co-cultivated with EHA105. Selection and regeneration of transformed cells and shoots of both cultivars was carried out for 12 weeks on selection medium containing 50 mg/L kanamycin and 250 mg/L timentin. The selection media were Quoirin and Lepoivre (1977) medium supplemented with 0.5 mg/L benzylaminopurine (BA) + 0.05 mg/L indole-3-butyric acid (IBA), and Woody Plant medium (Lloyd and McCown, 1980) containing 2.0 mg/L BA + 1.0 mg/L IBA for Montmorency and Gisela 6, respectively. Under optimal transformation conditions, 7 out of 226 (3.1%) inoculated leaf explants of cv. Montmorency and 5 out of 152 (3.9%) inoculated leaf explants of cv. Gisela 6 produced 30/39 GUS- and PCR-positive regenerants from the wounded midribs via an intermediate callusing stage after 12 weeks. The selected transformants have a normal phenotype in vitro. Southern blot analysis of the GUS- and PCR-positive transformants confirmed stable integration of the transgenes with 1-3 copy numbers in 5 lines of Montmorency and 7 of Gisela 6. This work demonstrates the potential of using *A. tumefaciens* to introduce and express foreign genes into cherry cultivars and rootstocks.

P-2065

Genetic Transformation of Canola (*Brassica napus* L.) for Engineering Resistance Using Gene Encoding Antimicrobial Peptides. K. GHASEMI BEZDI, V. S. Sheveloukha, and G. I. Karlov. Dept. of Agricultural Biotechnology, Moscow Timiryazev Agricultural Academy, Corpus 16, Timiryazevskaya St. Moscow 127550, RUSSIA. Email: kghasemibezdi@yahoo.com

An antimicrobial peptide with no significant amino acid sequence and low-molecular-weight has been isolated from the nut kernels of *Macadamia integrifolia* by A. M. McManus et al. in 1999 at the University of Queensland of Australia. They termed the peptide, MiAMP1, and suggested the purified MiAMP1 inhibited the growth of several microbial plant pathogens *in vitro* while having no effect on mammalian or plant cells. It is considered to be a potentially useful tool in genetic manipulations to increase disease resistance in transgenic crop plants. Therefore, we obtained a DNA sequence encoding MiAMP1 with an additional ATG start codon which was cloned into the cloning site of a pGEM-T vector (Promega) from Australia. This construction was then transformed to *E. coli* strain DH10B. PCR primers flanking the coding region of the MiAMP1 gene were engineered to contain restriction sites for *NcoI* and *BamHI*. These primers were then used to amplify the coding region of MiAMP1 cDNA. After excising DNA bands from agarose gels and purifying them using a DNA clean-up kit, the PCR-cloned fragment was digested using restriction enzymes *NcoI* and *BamHI* to release the MiAMP1 gene fragment. The binary vector p-CAMBIA1305.1 was digested with the restriction enzymes *NcoI* and *BglIII*. Subsequently, the two fragments were ligated using T4 DNA ligase to produce p-CAMBIA1305.1-MiAMP1 binary vector for plant transformation, that contains the full coding region of the MiAMP1 DNA flanked at its 5' end by the strong constitutive promoter of 35S from the cauliflower mosaic virus (CaMV). The coding region of MiAMP1 DNA is flanked at its 3' end by the polyadenylation sequence of NOS polyA. The plasmid also contains other elements useful for plant transformation such as a kanamycin resistance gene (*kan*), a hygromycin resistance gene (*hpt II*) driven by the CaMV35S double enhancer promoter and the GUS^{Plus} gene contains the intron to ensure detection of plant-specific glucuronidase expression. These and other features allow for selection in various cloning and transformation procedures. Also we optimized important parameters for regeneration of canola (*Brassica napus* L.). At the present time, we are carrying the canola transformation via *Agrobacterium* method using cotyledons as explant tissue of *B. napus* and co-culturing strains containing pCAMBIA1305.1-MiAMP1.

P-2066

Complete Chloroplast Genome Sequence of *Glycine max* and Comparative Analyses with Other Legume Genomes. Christopher Saski¹, SEUNGBUM LEE², Todd Wood¹, Jeffrey Tomkins¹, Hyi-Gyung Kim³, Robert K. Jansen³, and Henry Daniell². ¹Clemson University Genomics Institute, Biosystems Research Complex, 51 New Cherry Street, Clemson University, Clemson, SC 29634; ²University of Central Florida, Dept. of Molecular Biology & Microbiology, Biomolecular Science, Building #20, Orlando, FL 32816-2364; and ³Section of Integrative Biology and Institute of Cellular and Molecular Biology, Patterson Laboratories 141, University of Texas, Austin, TX 78712. Email: daniell@mail.ucf.edu

Lack of chloroplast genome sequences is still one of the major limitations to extending chloroplast genetic engineering technology to useful crops. Therefore, we sequenced the soybean chloroplast genome and compared it to the other completely sequenced legumes, *Lotus* and *Medicago*. The chloroplast genome of *Glycine* is 152,218 bp, including a pair of IR's of 25,574 bp of identical sequence separated by a SSC region of 17,895 bp, and a LSC region of 83,175 bp. The genome contains 111 unique genes and 19 of these are duplicated in the IR. Gene content of the three legumes is identical except that *Medicago* has lost one copy of the IR. The *rpl22* gene is missing from all three legumes and *Medicago* is also missing *rps16*. Gene order in *Glycine*, *Lotus*, and *Medicago* differs from the usual gene order for angiosperm chloroplast genomes by the presence of a single, large inversion of 51 kilobases (kb). Rates and patterns of sequence evolution among the three legume genomes indicate genes in the single copy of the IR in *Medicago* have evolved nearly two times faster than the same genes that are duplicated in *Lotus* and *Glycine*. Many of the *Glycine* repeats in the intergenic spacer regions and introns are found in the same location in the other legumes and in *Arabidopsis*, suggesting that they may play some functional role. The presence of small repeats of portions of *psbA* and *rbcL* in legumes that have lost one copy of the IR indicate that this loss has only occurred once during the evolutionary history of legumes.

P-2067

Effect of Simulated Wavelength Colors on Tissue Culture Regeneration and Development of Daylily (*Hemerocallis fulva* L.). JOHNNY CARTER and A. J. Askew. Agricultural Research Station, Fort Valley State University, Fort Valley, GA 31030. Email: carterj@fvsu.edu

During Summer 2004, a study was conducted to determine the effect of different and stimulated wavelength colors on tissue culture regeneration and development of daylily. The wavelength colors used were stimulated with cubical boxes constructed from colored transparency plastic sheets. Red, yellow, violet, green, blue, and a control were the stimulated wavelength color treatments observed. Each treatment was replicated four times in a complete randomized block design. Two cultivars ('E.L. Stout' and 'Amadeus') were used. Daylily explants were cultured on a MS + BAP (3.0 mg/L) + IAA (0.5 mg/L) medium under the stimulated wavelength colors. After six weeks of culture, the results showed that the two cultivars responded similarly to different light treatments. The yellow and violet treatments significantly promoted embryo regeneration and development when compared to the control. However, the red, green, and blue treatments significantly suppressed embryo regeneration and development

P-2068

In Vitro Cold Storage of Stone Fruit Germplasm in Kazakhstan. I. KOVALCHUK¹, S. Kushnarenko¹, B. Reed², and I. Rakhimbaev¹. ¹Laboratory of Biotechnology, Institute of Plant Physiology, Genetics and Bioengineering, 480090, 45 Timiryazev Str., Almaty, KAZAKHSTAN, and ²USDA-ARS National Clonal Germplasm Repository, 33447 Peoria Rd, Corvallis, OR 97333-2521. Email: kovalchuk_i_u@mail.ru

Duration of *in vitro* cold storage ($4 \pm 1^\circ \text{C}$, light intensity 7-15 $\mu\text{mol m}^{-2}\text{s}^{-1}$, 10-hour photoperiod) for stone-fruit shoots was studied. Twenty-five cherry, six sweet cherry and eight plum genotypes were cultured in glass tubes or in plastic air-penetrating bags on hormone-free Murashige and Skoog medium. Condition rating of stored shoots depended on genotype and type of storage container. Mean storage duration of sweet cherry and plum shoots was 9 to 12 months and cherry shoots ranged from 3 to 12 months. Storage of cultures in plastic bags was more effective than in glass tubes. Cherry shoots were tested for the effects of carbohydrates (sucrose and mannitol), growth regulators (BAP, IBA and ABA) and nitrate concentration (100%, 50%, and 25%) on cold storage duration. MS medium with 0.5 mg/l BAP, 0.1 mg/l IBA and 3% sucrose (or 2% sucrose + 2% mannitol) was optimal for all tested cherry genotypes and extended cold storage to 21 months or more. ABA did not influence cold-storage duration for any cherry genotypes. Nitrate concentration effects on cherry shoot survival were genotype dependent and there was no general trend.

P-2069

A Dual Color Fluorescence Staining Tool for the Assessment of Plant Cell Viability. K. OPPER, L. Ralston, and K. Song. Sigma-Aldrich Biotechnology, Research and Development, P.O. Box 14508, St. Louis, MO 63178. Email: LRalston@sial.com

The ability to determine cell viability is extremely important in cell and tissue culture as well as in studies of apoptosis and plant-pathogen interactions. Commonly used cell viability assays include trypan blue exclusion, ⁵¹Cr release, and ¹⁴C-labeled amino acid uptake and incorporation. These assays suffer from poor sensitivity, safety issues, and/or lengthy assay times. A preferable method for determination of cell viability is the differential staining of viable and non-viable cells by fluorescent dyes. Below, we describe a dual color fluorescent staining system for the identification of viable and non-viable plant cells. In optimizing this system, we screened multiple fluorescent dyes for their ability to specifically label viable or non-viable cells while exhibiting low photobleaching rates as well as minimal background fluorescence and non-specific cell wall staining. Using these criteria, we identified fluorescein diacetate and propidium iodide as the best dye combination for staining plant cells. We then went on to optimize dye concentrations and staining conditions for fluorescence microscopy. This system was optimized using tobacco cell suspension culture. However, we found this optimized staining system to work equally well on a variety of plant species and tissue types.

P-2070

A Comparison of Visual and Image Analysis for the Storage of Micro-propagated Plants. Hailu M. Aynalem¹, Timothy L. Righetti¹, and BARBARA M. REED². ¹Oregon State University, 4017ALS, Corvallis, OR 97331-7304 and ²US Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository, 33447 Peoria Road, Corvallis, OR 97333-2521. Email: corbr@ars-grin.gov

In vitro plants in slow-growth storage require routine evaluation for assessment of viability and timing of repropagation. Determination of plantlet health by visual assessment is difficult due to variations in growth pattern and plant structure. Developing a standardized system of plant evaluation would improve the efficiency of *in vitro* storage. A study was initiated to develop digital-image analysis techniques for plantlets during slow-growth storage and to compare that system with visual examinations. Pear, *Pyrus communis* L., cultivars were chosen for study because they have an open structure and clear position of internodes for image composition. Pear shoots stored at 4°C in tissue culture bags were evaluated monthly by visual examination and by digital image analysis. Digital images were evaluated for red, green, blue, NDVI (normalized differences of vegetation index), green/red (G/R), hue, intensity, and saturation (HIS) at the first two nodes of each plantlet. Over the first 5 months the visual ratings declined steadily for *P. communis*, 'Luscious' and 'Bartlett-Swiss' while 'Belle Lucrative' and 'Louise Bonne de Jersey' did not begin to decline until 9 months and then reached a plateau. Regression analysis indicated that the NDVI and G/R ratios changed significantly over the 15-month rating period for most cultivars. Correlations between visual ratings and G/R and NDVI values were significant ($R^2 = 0.5$) for 'Louise Bonne de Jersey', 'Luscious' and 'Bartlett-Swiss.' 'Belle Lucrative' visual ratings did not correlate with any color value data. These results will assist in the development of digital imaging as an alternative technique for evaluation of stored tissue culture plantlets.

P-2071

Production of Marker-free Wheat (*Triticum aestivum*) Plants Transformed by *Agrobacterium*. MANUELA CAMPA¹, Candida Vannini¹, Enrico Puja², Priscilla Francia², Maria Rosaria Stile², and Marcella Bracale¹. ¹D.A.S.S., Università degli studi dell'Insubria, via Dunant, 3, 21100, Varese, ITALY and ²ISTA S.P.A., Via San Martino, 20122, Milano, ITALY. Email: elasan@libero.it

Wheat (*Triticum aestivum*) is an important food crop, because of both the nutritional value of its seeds and the unique technological properties of flours prepared from those seeds. The high-molecular-weight glutenin subunits (HMW-GS), members of a family of seed storage proteins synthesized in developing wheat endosperm, are important determinants of the processing characteristic of wheat flours, for this reason, members of this gene family are important candidates for genetic engineering. Selectable marker genes are required to ensure the efficient genetic modification of crops. But agro-economic interests have prompted the development of several strategies to eliminate these genes from the genome after they have fulfilled their purpose. Recently, chemical-inducible Cre/loxP DNA recombination system (CLX) (1) have emerged and seem to provide a highly reliable method to generate marker-free transgenic *Arabidopsis* plants after transformation. We have used *Agrobacterium* strain AGL1 harbouring , as control plasmid, pX6-GFP, which contains the CLX system, *nptII* and *GFP*. Then, we have used the same strain harbouring the pX6-GFP based plasmid pX6-Glu, which contains also the gene *Dx5b* (coding for a HMW-GS). Till now, we have obtained 3 transgenic lines (cv. Bobwhite) transformed with the plasmid pX6GFP and 1 with pX6Glu, with a transformation frequency of 3.3-3.4% demonstrated by marker-gene expression and molecular analysis. We are performing chemical-induction tests on the immature embryos from these lines to obtain complete marker free plants: those transformed with pX6GFP expressing GFP and those transformed with pX6Glu expressing an increased amount of HMW-GS. 1. Zuo, J., Niu, Q.-W., Moller, S.G. & Chua, N.-H. Chemical-regulated, site specific DNA excision in transgenic plants. *Nat. Biotechnol.* 19, 157-161 (2001).

P-2072

Improving Digestibility of Forage Grasses by Genetic Manipulation of Lignin Biosynthesis. ZENG-YU WANG, Lei Chen, Xiao-Fei Cheng, Chung-Kyoon Auh, and Paul Dowling. Forage Improvement Division, The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401. Email: zywang@noble.org

Lignin is the major limiting factor for cell wall digestibility in forages. Feeding and grazing studies have shown that small changes in forage digestibility/degradability can have a significant impact on animal performance. Cinnamyl alcohol dehydrogenase (CAD) and caffeic acid *O*-methyltransferase (COMT) are key enzymes involved in lignin biosynthesis. CAD and COMT cDNA sequences were cloned from tall fescue, the predominant cool-season forage grass in the United States. Transgenic tall fescue plants carrying either sense or antisense CAD and COMT gene constructs were obtained by microprojectile bombardment of embryogenic suspension cells. Severely reduced mRNA levels and significantly decreased enzymatic activities were found in four transgenic lines. These CAD and COMT down-regulated tall fescue plants had reduced lignin content and altered lignin composition. No significant changes in cellulose, hemicellulose, neutral sugar composition, *p*-coumaric acid and ferulic acid levels were observed in the transgenic plants. In vitro dry matter digestibility increased by 7.2% to 10.5% in the transgenic lines, thus providing novel germplasm to be used for the development of grass cultivars with improved forage quality. This is the first report on improvement of dry matter digestibility in a cool-season forage grass by transgenic manipulation of lignin biosynthesis. Currently we are taking a similar approach to improve digestibility of bermudagrass, a widely grown warm-season grass.

P-2073

Effect of Sodium Butyrate on Production of Paclitaxel in *Taxus chinensis* Cell Culture. CHANG-HEON KIM, J. A. Kim, J. Y. Song, and H. J. Choi. Samyang Genex Food & Bio Research Center, Daejeon, 305-717, KOREA. Email: chkim@genex.co.kr

Sodium butyrate is known to be a potent inhibitor of histone deacetylase, and has been reported to induce apoptosis, cell growth arrest and expression of several genes. However, there have been no reports to date about effect of sodium butyrate on the stimulation of secondary metabolites in plant cell cultures. This work was conducted to improve production of paclitaxel in *Taxus chinensis* cell culture using sodium butyrate as a new type of elicitor based on its ability to induce histone hyperacetylation. Production of paclitaxel was increased about 2 fold when 1 mM of sodium butyrate was added to the culture at 7 days after incubation. Paclitaxel and other taxane production were further increased about 1.6 to 9 fold with repeated treatment of sodium butyrate. The effect of sodium butyrate on paclitaxel production was compared with other histone deacetylase inhibitors to examine possible role of sodium butyrate. These and other results suggest that sodium butyrate can be affecting secondary metabolism in plant cell culture via induction of histone acetylation. Further experiments are undergoing to elucidate the possible mechanism of sodium butyrate-mediated enhancement of secondary metabolites.

P-2074

Resistance to Tobacco Black Shank (*Phytophthora parasitica* var. *nicotianae*) in *Nicotiana* Species. W. T. Bass¹, P. L. Cornelius², W. C. Nesmith³, and B.-C. LI¹. ¹University of Kentucky, Kentucky Tobacco Research and Development Center, Lexington, KY 40546; ²University of Kentucky, Department of Agronomy, Lexington, KY 40546; and ³University of Kentucky, Department of Plant Pathology, Lexington, KY 40546. Email: bli2@uky.edu

As part of the effort to develop *Nicotiana*-based plant varieties for the production of plant-made pharmaceuticals (PMP), we evaluated 97 *Nicotiana* accessions from 37 species from the USDA collection and other sources for resistance to the major tobacco disease black shank. The roots of 26-day-old growth-room-grown plants were inoculated with zoospores of a local isolate of *Phytophthora parasitica* var. *nicotianae* race 1 (R1-LI1), the most damaging race for this fungus. The percentage of plants that were not infected 10-14 days after inoculation was used to determine resistance to the fungus. Among the 97 accessions evaluated, three accessions from *N. debneyi*, two accessions from *N. repanda*, and one accession each from *N. megalosiphon*, *N. plumbaginifolia*, *N. suaveolens*, and *N. sylvestris* were found to be resistant to R1-LI1. These nine accessions were further challenged by another local isolate of race 1 (R1-LI2) and a local isolate of race 0 (R0-LI1). All nine accessions, except for the *N. sylvestris* accession, were found to be resistant to both R1-LI2 and R0-LI1. We are therefore encouraged that undomesticated *Nicotiana* species constitute an excellent genetic source for resistance to the major tobacco disease black shank.

P-2075

Conservation of Turk's-Cap Lily (*Lilium martagon* L.) Bulblets in Hypoxic and Hypothermic Conditions. D. BOLBA, J. Roggemans, and A. Lebrun. Haute Ecole Charlemagne, Laboratory of Plant Tissue Culture, Rue Verlaine 9, Be-5030 Gembloux, BELGIUM. Email: dorinabolba@yahoo.com

Medium term effective conservation (3, 6 and 12 months) was achieved by manipulating some factors allowing the slow growth of *in vitro* generated bulblets. The material was stored in various conditions: hypoxia by liquid paraffin overlay, at +24° C, 16 h light/ 8 h dark, and hypothermia (+4° C) in obscurity, with and without paraffin film. *In vitro* reaction of stocked bulblets (viability, weight) was tested post-conservation and by transferring the conserved material on special media. In order to check that bulblets leaflet cultivated in normal conditions preserved their biosynthetic capacities, chlorophylls and total proteins were assessed. The exposure to hypothermia and hypoxia for 6 and 12 months affected the viability, but the minimal percentage decreased to half after 1 year of storage. The conservation conditions acted significantly as growth retarding factors for weight variations (dry weight substance accumulations) for the 6 and 12 months of storage. Regenerative processes increased significantly after transfer to normal culture conditions. The hormonal balance of transfer media was stimulating and specific. The assessed biochemical parameters at bulblets leaflet level demonstrated that the conserved material regains the growing capacity to its cultivation in optimal conditions. We concluded that the biosynthetic process slowing down was evident, so demonstrating the efficiency of the studied techniques for Turk's-Cap Lily medium term-conservation.

P-2076

Somatic Embryogenesis and Plant Regeneration from Valeria (*Valeriana officinalis*), a Medicinal Plant. KIOFA CLARK, K. Knowles, and S. K. Dhir. Center for Biotechnology, Fort Valley State University, Fort Valley, GA 31030. Email: dhirs0@fvsu.edu

Valeria (*Valeriana officinalis* L.) is a hardy, perennial, flowering plant used as an herbal medicine. The roots contain a compound, Valerian, an excellent remedy for anxiety, nervous tension and insomnia. In order to produce Valerian on large scale a mass propagation method for Valeria is desirable. The population of this plant is decreasing drastically due to poor seed germination. Improvement of this plant by traditional breeding methods is labor intensive and time consuming. To date no *in vitro* study of this valuable medicinal plant has been reported. Therefore, a protocol using *in vitro* culture for somatic embryogenesis was established. Different explants such as shoot tip, buds, node and leaf segments were excised (into 9-12 segments each of 5-10 mm segments) and were cultured on Murashige and Skoog's (MS) media supplemented with combination of Benzyl amino purine (BAP), Naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA). The highest number of embryos of 25-35 was observed from shoot tip with medium supplemented with 1.0 mg/l and 0.1 mg/l after 4 weeks under dark conditions. Whereas node explants produced only 15-18 embryos. Other explants tested, produced no embryos and only roots or callus formation was observed. The regenerated shoot rooted either on all or half strength MS media containing various concentrations of NAA and Indole butyric acid (IBA).

P-2077

The Effects of Ethylene Inhibitors on Rooting of Three Endangered Florida Pawpaws *In Vitro*. M. A. JASKOWIAK, V. C. Pence, and S. Charls. Cincinnati Zoo and Botanical Garden, Center for Conservation and Research of Endangered Wildlife (CREW), Cincinnati, OH 45220. Email: mjaskowiak@cincinnati-zoo.org

Three federally endangered Florida pawpaws, *Asimina tetramera*, *Deeringothamnus pulchellus* and *D. rugelli*, have been propagated *in vitro* as part of the Endangered Plant Propagation Program. However, like many woody plants, these species are extremely difficult to root. Previous studies have suggested that a high level of ethylene acts as an inhibitor to rooting in these species. The pawpaws were first successfully rooted on WP medium with 3% sucrose, 0.22% Phytigel and 0.5 mg l⁻¹ IBA supplemented with 50-100 μM sodium thiosulfate (STS), an inhibitor of ethylene action, but rooting averaged only 18%. So, several other ethylene inhibitors were examined: 33 mg l⁻¹ silver nitrate (AgNO₃), another inhibitor of ethylene action; 10 μM aminovinylglycine (AVG), an inhibitor of ethylene production; and 1% charcoal, which absorbs ethylene from the medium, used after a 24 hr pulse of 50 or 100 mg l⁻¹ IBA. AgNO₃ and AVG were added to media with 0.5 or 1.0 mg l⁻¹ IBA. Rooting on STS and AgNO₃ averaged 19% and 16%, respectively, and with a pairwise t-test, there was no statistical difference between the two treatments (p=.0458). AVG has also produced some rooting, but contamination rates in all AVG experiments have been high. Rooting also occurred on charcoal medium after a pulse of IBA, but at a rate similar to that seen on other media. The use of ethylene inhibitors stimulates rooting in these species, whereas no rooting has been observed on unsupplemented media. However, rooting in these plants appears to be affected by other factors in addition to high levels of ethylene. Further studies are needed to identify these, as well as to determine the most effective ethylene inhibitor for these species. This research was funded in part by a grant from the Institute of Museum and Library Sciences and is in collaboration with the Center for Plant Conservation and Historic Bok Sanctuary.

P-2078

In Vitro Flowering and Differential Accumulation of Andrographolide in Cultures of *Andrographis lineata* Nees - an Important Medicinal Plant of India. ARIFULLAH MOHAMMED and Ghanta Rama Gopal. S. V. University, Dept. of Botany, Tirupati, AP 517502, INDIA. Email: maarif68@rediffmail.com

The transition in plants from the vegetative state to reproductive development is of great interest to botanists but is still poorly understood. Under natural growth conditions flower formation usually commences when a plant attains maturity. The age of the plant is genetically controlled and is species specific. The plant flowers only when genetic factors including photoperiod response are congenial. These conditions can often be altered so that the plant can be induced to undergo an early reproductive phase. Such an attempt to induce flowering *in vitro* from juvenile explants of *Andrographis lineata* (Acanthaceae) is reported here. *De novo* flower morphogenesis was induced on explants from both mature and seedling plants on Murashige and Skoog (MS) medium fortified with 6-benzyladenine (BA) and indole-3-acetic acid (IAA). Highest percentage of flowering was achieved with mature nodal explants on MS medium supplemented with 2 mg l⁻¹ BA + 2 mg l⁻¹ IAA + 0.01% Polyvinyl pyrrolidone (PVP). Multiple flower bud induction was found on cotyledonary node cultured on aforesaid medium. Flower buds were also noticed to be formed on explants cultured in liquid medium. *A. lineata* is used by traditional medical practitioners as mucolytic agent to treat all respiratory infections. The main active principle, Andrographolide (C₂₀H₃₀O₅), was isolated from *A. lineata* (yield 8.75%), which is extensively used in pharmacopy. It has been established that, during budding and early flowering stages, the basil oil was rich in monoterpenes and Xanthotoxin in furanocoumarins. Hence, we estimated Andrographolide (a diterpene lactone) content of *in vitro* cultures by HPLC methods. Distinct patterns of Andrographolide represented by different tissue types were observed, suggesting tissue specific accumulation of Andrographolide in respective tissues. On the whole, the protocol developed in this study provides a system for induction of flowering and monitoring of Andrographolide content at various stages of *in vitro* cultures of *A. lineata*.

P-2079

Engineering Cytoplasmic Male Sterility via the Chloroplast Genome. Oscar N. Ruiz and HENRY DANIELL. Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science Bldg. # 20, Room 336, Orlando, FL 32816-2364. Email: daniell@mail.ucf.edu

While investigating expression of the polyhydroxybutyrate pathway in transgenic chloroplasts, we addressed the specific role of β-ketothiolase. Therefore, we expressed the *phaA* gene via the chloroplast genome. Prior attempts to express the *phaA* gene in transgenic plants were unsuccessful. We studied the effect of light regulation of the *phaA* gene using the *psbA* promoter and 5' untranslated region (5'UTR), and evaluated expression under different photoperiods. Stable transgene integration into the chloroplast genome and homoplasmy were confirmed by Southern analysis. The *phaA* gene was efficiently transcribed in all tissue types examined, including leaves, flowers and anthers. Coomassie-stained gel and western blots confirmed hyperexpression of β-ketothiolase in leaves and anthers, with proportionately high levels of enzyme activity. The transgenic lines were normal except for the male sterile phenotype, lacking pollen. Scanning electron microscopy revealed a collapsed morphology of the pollen grains. Floral developmental studies revealed that transgenic lines showed an accelerated pattern of anther development, affecting their maturation and resulted in aberrant tissue patterns. Abnormal thickening of the outer wall, enlarged endothecium and vacuolation, decreased the inner space of the locules, affected pollen grain and resulted in the irregular shape or collapsed phenotype. Reversibility of the male sterile phenotype was observed under continuous illumination, resulting in viable pollen and copious amount of seeds. This study resulted in the first engineered cytoplasmic male sterility system in plants, offers a new tool for transgene containment for both nuclear and organelle genomes and provides an expedient mechanism for F1 hybrid seed production.

P-2080

Metabolic Engineering of the Chloroplast Genome Using the *E. coli* *ubiC* Gene Reveals that Chorismate is a Readily Abundant Plant Precursor for p-Hydroxybenzoic Acid Biosynthesis. ANDREW L. DEVINE¹, Paul V. Viitanen², Muhammad Sarwar Khan²⁺, Deborah L. Deuel², Drew E. Van Dyk², and Henry Daniell¹. ¹Department of Molecular and Microbiology, University of Central Florida, Biomolecular Science Building #20, Room 336, Orlando, FL 32816-2360 and ²DuPont Experimental Station, Building 402/2237, Rt. 141 and Henry Clay, Wilmington, DE 19880-0402. Email: daniell@mail.ucf.edu

p-Hydroxybenzoic acid (pHBA) is the major monomer in liquid crystal polymers. In the present study the *E. coli* *ubiC* gene that codes for chorismate pyruvate-lyase (CPL) was integrated into the tobacco chloroplast genome under the control of the light-regulated *psbA* 5' untranslated region. CPL catalyzes the direct conversion of chorismate—an important branch point intermediate in the shikimate pathway that is exclusively synthesized in plastids—to pHBA and pyruvate. The leaf content of pHBA glucose conjugates in fully mature T1 plants exposed to continuous light (total pooled material) varied between 13-18% DW, while the oldest leaves had levels as high as 26.5% DW. The latter value is 50-fold higher than the best value reported for nuclear-transformed tobacco plants expressing a chloroplast-targeted version of CPL. Despite the massive diversion of chorismate to pHBA, the plastid-transformed plants and control plants were indistinguishable. The highest CPL enzyme activity in pooled leaf material from adult T1 plants was 50,783 pkat/mg of protein, which is equivalent to ~35% of the total soluble protein and ~250 times higher than the highest reported value for nuclear transformation. These experiments demonstrate that the current limitation for pHBA production in nuclear-transformed plants is CPL enzyme activity, and that the process becomes substrate-limited only when the enzyme is present at very high levels in the compartment of interest, such as the case with plastid transformation. Integration of CPL into the chloroplast genome provides a dramatic demonstration of the high-flux potential of the shikimate pathway for chorismate biosynthesis, and could prove to be a cost-effective route to pHBA. Moreover, exploiting this strategy to create an artificial metabolic sink for chorismate could provide new insight on regulation of the plant shikimate pathway and its complex interactions with downstream branches of secondary metabolism, which is currently poorly understood.

P-2081

Biolistic Transformation of Easter Lily is Dependent on Callus Type. K. KAMO¹ and B. H. Han². ¹Floral & Nursery Plants Research Unit, National Arboretum, USDA, Beltsville, MD 20705 and ²Horticulture Research Institute, Rural Development Administration, Suwon, REPUBLIC OF KOREA. Email: kamok@ba.ars.usda.gov

Two types of callus, a fast-growing friable callus and a slow-growing compact callus, were initiated from bulb scales of *Lilium longiflorum* cv. Nellie White. Sucrose, 3-12%, affected induction of friable callus but not compact callus. Friable callus was initiated when bulb scales were cultured on MS medium with 2 mg/L dicamba and 9% sucrose. Friable callus grew 5× faster than compact callus. There were many more plants regenerated from compact callus than friable callus, and these plants were phenotypically normal when grown to the flowering stage. Friable callus formed numerous somatic embryos when the callus was cultured on MS basal salts medium with 1% activated charcoal, but only a few somatic embryos germinated to form plants. Regeneration from compact callus appeared to be by organogenesis. Transformation was never achieved using friable callus that had been bombarded with either *bar* and *uidA* genes under the CaMV 35S promoter or *nptII* and *uidA* genes under the CaMV 35S promoter. One month following bombardment of friable callus, the number of gus positive cells declined significantly. Transformed plants were selected from compact callus bombarded with these same plasmids indicating the benefit of using compact callus for transformation of Easter lily.

P-2082

Transformability of *Nicotiana* Species by *Agrobacterium tumefaciens*: Using a Transient Approach. BAOCHUN LI, Hui Qiu, Huan Xie, and Troy Bass. Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY 40546-0236. Email: bli2@uky.edu

Transformability is an essential trait for plant varieties to be used for the production of plant-made pharmaceuticals (PMP). As part of the effort to develop plant varieties optimal for PMP production, this study investigated the transformability of diverse *Nicotiana* species by *Agrobacterium tumefaciens* using a transient approach. Leaf explants derived from one-month-old seedlings of in-vitro-grown *Nicotiana* plants were infected by *A. tumefaciens* GV3850 carrying a binary vector that harbors a *gusA* gene and an *nptII* gene. The infected leaf explants were incubated for two days before they were subjected to *gusA* histochemical assay. The transformability was determined as the percentage of leaf explants expressing the *gusA* gene and as the intensity of *gusA* expression per responsive leaf explant. We are now in the final stage of evaluating 131 *Nicotiana* accessions from 55 species from the USDA collection and other sources. The transformability of these *Nicotiana* accessions will be presented.

P-2083

Stability of β -glucuronidase Expression in Vegetative Generations of Transgenic *Hevea brasiliensis*. P. AROKIARAJ¹, R. Leelawathy¹, and S. Siti Hawa². ¹The Malaysian Rubber Board, Rubber Research Institute of Malaysia Experimental Station, Biotechnology and Strategic Research Unit, 47000 Sungei Buloh, Selangor, MALAYSIA and ²The Malaysian Rubber Board, Rubber Research Institute of Malaysia, Information Technology Unit, Bangunan Getah Asli, 148 Jalan Ampang, 50908 Kuala Lumpur, MALAYSIA. Email: parokiaraj@lgm.gov.my

Field testing showed that the *uidA* cDNA that encodes for the enzyme β -glucuronidase was stably expressed in latex of four transgenic GUS plants with three generations (V1, V2 and V3), which were derived vegetatively from a single transgenic GUS mother plant. Histochemical analysis revealed a blue colouration of the latex when incubated in x-gluc from all transformants containing the β -glucuronidase protein (GUS). GUS enzyme activity in latex of transgenic rubber plants was also analysed by fluorometry, average GUS activity ranged from 74 to 93 pmol 4- μ /min/mg protein between the plants analysed. Likewise, the average GUS activity between generations (V1, V2 and V3) ranged from 75 to 94 pmol 4- μ /min/mg protein. Control plants in all cases gave a background reading of less than 2 pmol 4- μ /min/mg protein. Upon statistical analysis, results showed no significant differences in both analysis. The P-value showed 0.8565 for GUS expression between plants and 0.6095 between generations. The *uidA* copy number, based on SYBR green I detection were determined in vegetative generations using genomic DNA. Using the resultant linear relationships for p35SGUSINT as standard curves, the copy number of the *uidA* insert was determined as a single copy insert in all vegetatively propagated transgenic plants analysed.

P-2084

Characterization of Heterologous Multigene Operons in Transgenic Chloroplasts: Transcription, Processing and Translation. TANIA QUESADA-VARGAS, Oscar N. Ruiz, and HENRY DANIELL. Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science Bldg. # 20, Room 336, Orlando, FL 32816-2364. Email: daniell@mail.ucf.edu

Although transcription, post-transcriptional processing and translation have been previously studied in the chloroplast, little is known about these processes when heterologous operons are introduced in the chloroplast genome. This work reports, for the first time, a detailed characterization of these processes in transplastomic tobacco lines harboring seven different heterologous operons. Northern blot analyses revealed that, generally, polycistronic mRNA was the only transcript produced, and that processing into monocistrons prior to translation did not occur. Foreign protein accumulation was detected in these transgenic lines, indicating abundant polycistron translation. Polycistron processing was only detected when linked to the presence of heterologous bacterial sequences, which formed stable stem-loop structures. This indicates that the chloroplast posttranscriptional machinery is able to recognize sequences that are not of chloroplast origin. However, not all stable stem-loop structures were processed, suggesting that other mechanisms additional to the presence of secondary structures may be involved. Both processed and unprocessed heterologous polycistrons were stable, even in the absence of 3' untranslated regions (UTRs). Unlike native 5'UTRs, heterologous secondary structures of 5'UTRs showed efficient translational enhancement independently of cellular control. Furthermore, abundant read-through transcription was observed in the presence of chloroplast 3'UTRs. Studies involving polyribosome fractionation assays are currently in process to provide further evidence to the above observations. Addressing questions about polycistrons, and the sequences required for processing and transcript stability is essential in chloroplast metabolic engineering. Knowledge of such factors would enable engineering of foreign pathways independently of the chloroplast complex post-transcriptional regulatory machinery.

P-2085

Factors Influencing GUS Transient Expression After Bombardment of Leaves of Rapid-cycling Fast Plants. M. YOUNG, G. Jones, S. Cogbill, T. Faulcon, K. Green, L. Chambers, M. McDaniel, G. Harmon, and R. Blackmon. Department of Biology, Elizabeth City State University, Elizabeth City, NC 27909. Email: mmyoung2@mail.ecsu.edu

Rapid-cycling fast plants (*Brassica rapa*: RCB_r) is a widely used model system for education in plant sciences and research. It has a very short generation time (seed-to-seed in less than 40 days), many mutants are available and physiological distresses are easily observed. However, there are few studies on transformation in this system. There are two main ways of introducing genes into plants: biolistics and *Agrobacterium tumefaciens*. Biolistics, the process of shooting DNA into cells/tissues using a gene gun, has the unique advantage of being genus and species independent. However, a major problem associated with this technique is the time required to optimize all the parameters involved in the delivery of the DNA. Important parameters that were optimized in this study included target pressure, target distance, amount of DNA and number of shots. Seeds of RCB_r were surface sterilized in 10% sodium hypochlorite and germinated in the dark on Murashige and Skoog-based basal medium. After three days, the seedlings were transferred to the same medium and placed under 16 hour photoperiod. Aseptically grown leaves were excised and placed on medium containing BA and NAA one day prior to bombardment. A plasmid vector (pCAMBIA) containing the *gus* gene was extracted from *E. coli*, adhered onto gold particles and shot into the leaf tissues varying the parameters outlined earlier. Two days after bombardment, these leaves, along with controls, were assayed histochemically for the GUS protein. The implications for transformation of rapid-cycling fast plants will be discussed.

P-2086

Organogenic Calluses as Target Explants for *A. tumefaciens*-mediated Transformation in Soybean. Haiping Hong, HONGYI ZHANG, Helke Hillebrand, Todd Jones, and Ming Cheng. BASF Plant Science GmbH, Agricultural Center BPS - Li 554, D-67114 Limburgerhof, GERMANY. Email: zhangh@basf.com

A regeneration and transformation system has been explored in soybean using organogenic calluses as the starting explants. Leaf-node or cot-node explants prepared from 7~8 d old seedlings were cultured on callus induction medium (CI) containing either Murashige and Skoog (MS) salts or modified FNL salts and B5 vitamins supplemented with various concentrations of benzyladenine (BA) and thidiazuron (TDZ) for 10 to 21 d. Calluses were then excised from the original explants and placed to fresh callus induction for callus proliferation. The proliferated calluses or the freshly excised calluses from the original explants were cut into small pieces (0.3~0.5 mm) and transferred to shoot induction medium (SI) containing MS salts and B5 vitamins supplemented BA and cultured for 30~60 d. After shoot primordia formed, tissues were transferred to shoot elongation medium (SE) containing MS medium supplemented with indole-3-acetic acid (IAA), zeatin riboside, and gibberellic acid (GA). Elongated shoots (2~3 cm) were obtained after 30~60 d culture on the medium. All subcultures were performed with a 2~3-week interval. Elongated shoots were excised from the explants and rooted in rooting medium containing half-strength B5 salts and vitamins supplemented with IBA. Plant regeneration was demonstrated in multiple elite soybean genotypes. Both the primary callus and the proliferated callus were used as target explants for *Agrobacterium*-mediated transformation. The calluses were infected with *A. tumefaciens* harboring a binary vector with the bar gene as the selectable marker gene and the *uidA* gene for GUS expression. Usually 60~100% of the callus showed transient GUS expression 5 d after inoculation. Infected calluses were then selected on CI, SI or SE medium amended with various concentrations of glufosinate. To date, multiple transgenic soybean plants have been regenerated and established in the greenhouse. Strong GUS expression was exhibited on various tissues such as leaf, stem and roots. Transformation was confirmed by TaqMan analysis.

P-2087

Bio-efficacy of an Alkaloid and Flavonoids from *Solanum dulcamara*. L. P. KUMAR and S. Bhaduria. Laboratory of Plant Tissue Culture and Secondary Metabolites, Department of Botany, University of Rajasthan, Jaipur-302004, INDIA. Email: godgift1955@yahoo.co.in

Dermatophytoses poses a serious concern to the sociologically backward and economically poor population. Mycosis, though normally not lethal are unpleasant and difficult to cure. Superficial infections caused by keratinophilic fungi are called ringworm infection or Tinea infection. The disease is predominant in tropical and subtropical countries due to their prevailing moisture and temperature regimes and is a problem inspite of several antimycotic drugs available in the market. Similarly problems are due to bacterial diseases. Due to various side effects and high cost of synthetic drugs, interest has been developed in the use of herbal medicines which have been reported to have either very little or no side effects. In the present study, attempts have been made to extract and study the antidermatophytic and antibacterial activity of an alkaloid and flavonoids respectively from *Solanum dulcamara*. The extracted alkaloid was screened against four pathogenic fungi viz *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum gypseum* and *Candida albicans*. The alkaloid showed better antidermatophytic activity against all four pathogens as compared to their standard drugs. Similarly free and bound flavonoid extracts from *Solanum dulcamara* were screened against four pathogenic bacteria viz & *Escherichia coli*, *Enterobacter aerogens*, *Proteus mirabilis* and *Staphylococcus aureus*. Bound flavonoids showed better results when compared with free flavonoids and results were nearly the same or better than the standards, specifically in case of *S. aureus* where the standard showed no activity, but much higher efficacy was seen in bound flavonoid extract.

P-2088

Development of a Regeneration Technology for *Prunus salicina* for Plum Pox Virus Resistance. L. TIAN and S. Sibbald. Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, London, Ontario, CANADA. N5V 4T3. Email: tianl@agr.gc.ca

Plum pox is a viral disease that affects stone fruits, including plum, peach, nectarine, cherry, and apricot. The disease was first reported in Eastern Europe and has gradually spread to many other countries. PPV disease causes significant damage to *Prunus* fruit trees and can result in significant economic losses to fruit production. Few naturally occurring resistance sources have been found which can be used to develop highly resistant fruit varieties. Alternatively, genetic resistance to plum pox virus can be developed via introducing molecularly-designed resistance into *Prunus* species via genetic engineering. We started a research to develop plum pox virus resistance in *Prunus* species via biotechnology. Here we report a regeneration technology for *Prunus salicina*, an important *Prunus* species. Embryonic axes of seeds were sliced into 2 to 3 sections which were placed on a medium containing indolebutyric acid and thidiazuron. One month after on the medium, shoots were induced from the explants. Induced shoots were transferred to plant growth regulator-free medium. Shoots elongated and plantlets formed on the new medium. Upon transfer to soil, full plants were recovered in a greenhouse. Transformation technology based on established regeneration system is being developed.

P-2089

Free Polyamines During Acclimatization of Micropropagated Banana (*Musa* sp.). J. L. CASAS, M. D. Serna, F. Serrano, J. Ramirez, M. Cano, and A. Piqueras. Unidad de Biotecnología Vegetal, Instituto Universitario de Investigación CIBIO (Centro Iberoamericano de la Biodiversidad), Universidad de Alicante. Carretera de San Vicente del Raspeig s/n 03690 San Vicente del Raspeig, Alicante, SPAIN. Email: j.l.casas@ua.es

Many studies have analyzed the possible role of polyamines in organogenesis, somatic embryogenesis and rhizogenesis. However, very scarce information has been collected so far about the possible active role of these compounds in the critical phase of acclimatization from *in vitro* to *ex vitro* conditions. In this study we analyzed the acclimatization of micropropagated banana plants from the view of polyamine titer and pattern evolution in leaves and roots. Free polyamines were extracted and quantitated in *in vitro* plants and throughout acclimatization by weekly sampling of leaf and root tissue until week 6 in which plants were considered fully acclimatized. Putrescine, cadaverine, spermidine and spermine were all present in leaf tissue along acclimatization. However in roots from the same plants we only detect the four amines in *in vitro* plants and throughout the rest of the acclimatization period under study spermine was not detected. The presence of cadaverine in banana leaves was especially relevant because it was the major amine found at all stages studied apart from acclimatized plants. The process of acclimatization brought about the following main changes: - In leaves: ● Total polyamine content increased during the first two weeks of acclimatization (up to 5 $\mu\text{mol g}^{-1}$ dry weight) and then drastically decreased to 1,9 $\mu\text{mol g}^{-1}$ dry weight in fully acclimatized leaves. ● The diamine/polyamine ratio also changed significantly, being 3,8 in *in vitro* leaves and 0,6 in acclimatized leaves. - In roots: ● Changes in polyamine titer were already evident at the first week of acclimatization when polyamine content decreased to a 43,7% of that found in *in vitro* plants. ● As in leaves, diamine/polyamine ratio was very high (4,3) in *in vitro* plants and much lower (1,48) in acclimatized plants. In accordance with the results obtained we proposed that polyamines act as key compounds in controlling the cellular homeostasis of plant cells under the special features that imposes the *in vitro* environment and throughout the critical period of acclimatization to *ex vitro* conditions.

P-2090

Histological Changes in the Root Apex of Micropropagated Rosa Plantlets During Acclimatization. S. Hussein, E. Olmos, E. Hellín, and A. PIQUERAS. Dept. Plant Breeding, CEBAS (CSIC), PO Box 164.30100 Espinardo, Murcia, SPAIN. Email: piqueras@cebas.csic.es

During acclimatization to *ex vitro* conditions of micropropagated Rosa plantlets, several changes in the anatomy of *in vitro* formed root apex (first 500 micres). Histological examinations of both longitudinal and transversal sections of the root apex revealed a series of progressive structural and developmental changes in the root cap, the apical meristem and the surrounding tissues. Before acclimatization, *in vitro* formed roots presented an anomalous structured root cap meristem and cortex with large intercellular spaces similar to lysigenous aerenchima. Border cells at the periphery of the root cap did not secrete mucilage and only two lines of statocytes could be observed. At day seven of acclimatization to *ex vitro* conditions, significant histological changes towards normal development in the root apex were observed with a more structured apical meristem including a clearly developed quiescent center and a progressive reduced aerenchyma in the cortex. When the root apex was observed at day fifteen of acclimatization, its histological structure was almost normalized without aerenchyma in the cortex and several lines of statocytes. The apical meristem was clearly defined and differentiated from cortex and root cap. At the periphery of the root cap, border cells containing mucilage and the different stages of transition from statocysts to them could be observed. During the acclimatization to *ex vitro* conditions, the histology structure of the root apex reflects some of the adaptations of this vital part of the root system to the new environmental conditions of increased mechanical impedance, reduced humidity, increased aeration and heterogeneous distribution of nutrients which are required for the survival of the plant to *ex vitro* conditions. A precise evaluation of root apex developmental progression during acclimatization could improve current transfer to *in vitro* conditions being used in quality control of micropropagated plants, environmental conditions and the effectiveness of the different substrates.

P-2091

Morpho-histological Analysis of Key Developmental Stages During Somatic Embryogenesis in Saffron. S. Blázquez, E. Olmos¹, J. A. Fernández, and A. PIQUERAS¹. Plant Biotechnology Lab, IDR (UCLM), Campus de Albacete, 02071 Albacete, SPAIN and ¹Plant Breeding Dept. CEBAS (CSIC). PO Box 164, 30100, Espinardo, Murcia, SPAIN. Email: piqueras@cebas.csic.es

Nodular embryogenic calli were developed from cultured meristematic tissue of dormant saffron corms. Under aseptic conditions, a cubic section of the central meristem was cultured in MS medium supplemented with 2,4 D (0,1 mg/l) and BA (2, mg/l). Under these conditions the generation of nodular embryogenic calli differentiating somatic embryos could be observed after 6 weeks in culture. The emergent embryogenic calli have been subcultured each six weeks for the last 5 years remaining embryogenic so far. The generation of the somatic embryos in the calli was increased 5 times by using NAA instead 2,4 D and rising the concentration of BA to 2,5 mg/l. As a previous step to improve the regeneration of saffron plants via somatic embryogenesis, the ontogenic developmental stages (st) of this process have been characterized using light and electron microscopy. The ultrastructural morphological study of the embryogenic nodular calli at initial stages of differentiation, revealed an internal structure composed of cell clumps with meristematic morphology located at the surface of the embryogenic callus or surrounded by parenchymatic tissue. The meristematic cells presented a dense cytoplasm and thickened cell wall situated at the periphery of the embryogenic callus that underwent internal segmented divisions. Histological sections under light microscopy showed how continued divisions formed globular embryos that were attached to the callus surface by a broad multicellular structure (st 1). Further development of the embryoids was characterized by the emergence of a small apical meristem and a cotyledon (st 2) and subsequent differentiation of dipolarization (st 3) at the basal part of the embryo. Fully developed somatic embryos were separated from the embryogenic callus and presented a well developed apical meristem with a cotyledon at one end and a small rooted basal structure at the opposite end (st 4). The histological observations confirmed the embryogenic nature of the cultured nodular calli of saffron used in this study and that in this system, the plantlets were regenerated through somatic embryogenesis.

P-2092

In Silico Designing of the Hypothetical SMC Proteins Involved in Meiosis of *Arabidopsis thaliana*. Janet He², Sachin Gandhi², and VARSHA RAJA¹. ¹Department of Botany, University of Toronto, Toronto, Ontario M5S 3B2, CANADA and ²Xintra, Bioinformatics, Scarborough Town Center, Scarborough, Ontario, CANADA. Email: globalbiotech@care2.com

Identifying a protein's structure is a key to understand its biological function. Chromosome-associated proteins largely determine the structure of chromosomes. Members of the SMC (Structural Maintenance of Chromosomes) family play an important role in both prokaryotic and eukaryotic chromosome structure and its dynamics. SMC proteins are involved in chromosome condensation, sister-chromatid cohesion, sex-chromosome dosage compensation, genetic recombination and DNA repair. In eukaryotes, two different SMC protein complexes, condensin and cohesin, regulate chromosome condensation and sister chromatid cohesion, respectively. The SMC proteins are physically associated with chromosomes, suggesting that they may be part of the machinery that regulates local and global structural changes of chromosomes required for these functions. The subunits of the cohesin complex also share important links with proteins required for cohesion during meiosis, which seems to be highly conserved. We have used Bioinformatics tools to predict SMC proteins in *Arabidopsis thaliana*, including 3D, secondary, tertiary structures, structure superimposition, and active sites-, which combine with ligands. Discovery of the functions of the proteins produced by a model species will offer much information about the roles of proteins in all higher plants. There have been major advances recently in understanding the function of SMC proteins--including the identification of biochemical activities of SMC-containing protein complexes and the realization that individual SMC proteins might link seemingly unrelated aspects of chromosomal metabolism. Structural homology to other known structures and motifs was carried out by structural proteomics. Active sites for these meiotic proteins were identified by Automatic collection of protein sequence motifs. Choosing significant homolog SMC proteins, Motifs discovery and phylogenetic analysis, its possible functional and structural role thus was assigned.

P-2093

Iranian Olive Cultivars Micropropagation. FARZANEH TABESH¹, M. Peyvandi², and S. M. Hosseini-Mazinani³. ¹Dept. of Horticulture, Azad University of Science and Research, Tehran, IRAN; ²Dept. of Biology, Faculty of Science, Azad University, North Tehran Branch, IRAN; and ³National Research Center of Genetic Engineering and Biotechnology (NRCGEB), Tehran, IRAN. Email: fg_tabesh@yahoo.com

Commercial olive proliferation requires efficient methods and optimized protocols for mass scale multiplication. This paper deals with suitable protocols for proliferation of two Iranian olive cultivars, "*Dezful*" and "*Zard*". The main objective of this research is to determine a suitable medium and carbohydrate source for both cultivars. To this end, initially, auxiliary buds of young branches from 3 years old trees were cultured in OM and DKW media containing sucrose or mannitol (18 and 30 g/l) supplemented with 2ip (4 mg/l). Then, the uninodal explants were subcultured in the OM or DKW media supplemented with 2ip (4 mg/l), containing sucrose or mannitol (30 g/l). The results indicated that (i) at the primary stage of culture, for *Dezful* cultivar, DKW medium with sucrose provided the best results, while in *Zard* cultivar OM medium containing sucrose were better than the other media, (ii) in both cultivars, the existence of mannitol in the subculture media increased the growth of micro shoots, and (iii) micro shoots growth of *Dezful* cultivar in DKW and *Zard* cultivar in OM provided better results. Also the results indicated that different cultivars had different responses to the same conditions. Overall results suggested that although sucrose in the first stage of culture caused better growth of shoots, the explants that were grown in the mannitol from the beginning, were stronger and could be used for long term multiplication.

VT-2000

Growth Response of Mouse Mammary Carcinoma Cells in Presence of Anastrozole, Tamoxifen and the Combination of the Two Drugs. S. K. MAJUMDAR, J. M. Xanthopoulos, and A. E. Romano. Department of Biology, Lafayette College, Easton, PA 18042. Email: majumdas@lafayette.edu

For over two decades, tamoxifen, an antiestrogen, has been employed in the treatment of estrogen receptor positive advanced stage breast cancer. However, due to tamoxifen's partial agonistic effect on some hypersensitive breast cancer, other breast cancer treatment alternatives like anastrozole, an aromatase inhibitor have been introduced. In this report 4T1 murine mammary carcinoma cells grown both *in vitro* and *in vivo* were treated with the drugs individually or in combination. When injected into Balb/c mice, 4T1 cells rapidly multiply resulting in highly metastatic tumors, which closely resemble human breast cancer and serve as an animal model for stage IV breast cancer. *In vitro* efficacies were determined utilizing cell kinetics, a quick cell proliferation assay, and a mitochondrial membrane potential assay while *in vivo* treatment effects were evaluated by monitoring tumor development and mortality in female Balb/c mice. Although most drug inhibitory effects on cell multiplication were observed in the combination treatment, both anastrozole and tamoxifen individually inhibited cell multiplication significantly at most time periods in this mouse breast cancer cell line. The mitochondrial membrane potential disruption assay demonstrated a significant increase in percent of cells undergoing apoptosis in all treatment groups. However, the combination treatment was most effective in inducing cell death via apoptosis. In the *in vivo* tumor development study, tamoxifen and combination treated mice showed an increase in mouse life span when compared to anastrozole and untreated mice. Although all drug treatments extended the mean life span of the 4T1 inoculated tumor bearing mice, only tamoxifen and combination treatments statistically increased the life span when compared to untreated mice. This discrepancy may be the result of an increased estrogen presence in the *in vivo* setting compared to the *in vitro* environment.

VT-2001

Comparison of the Gene Expression of Interleukin-1 β -converting Enzyme (ICE) and the Cytokine Interleukin 18 (IL-18) Following Acute Lung Injury. DEREK TRUYEN PHAM¹, Ren-Feng Guo², Peter A. Ward², and Jacqueline A. Jordan¹. ¹Department of Natural Sciences, Clayton College & State University, Morrow, GA 30260 and ²Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109. Email: csu14570@mail.claytonstate.net

Over the years, lung inflammation has presented a challenge due to problems in identifying the mechanisms causing injury. Many cytokines are secreted during preliminary stages of inflammatory injury that initiate severe responses by the body's immune system. The focus of this study was to evaluate the role of IL-18 and ICE following acute lung injury. Previous research studies have shown that the gene expression of IL-18 increases following exposure to LPS (Lipopolysaccharide) and Immune Complex Injury (IC-IgG) in the adult rat lungs. These studies concluded IL-18 is an important pro-inflammatory cytokine that is involved in mediating lung injury. The activation of IL-18 is thought to be dependent on the converting enzyme ICE. ICE cleaves IL-18 from its precursor (pro-IL-18) to a mature activated form. We compared the gene expression of IL-18 and ICE in treated rat lungs. Total RNA was isolated from injury lungs and cDNA primers designed to monitor the expression IL-18 and ICE. Experimental results suggest a change in the expression of ICE is proportional to the levels of IL-18 following acute lung injury. Previous studies have shown that IL-18 is produced by alveolar macrophages. *In vitro* expression of ICE and IL-18 by activated macrophages is also under investigation. In summary, the modulation of ICE may also be a key regulatory of acute lung injury.

VT-2002

Identification of Alpha-actinin as a Putative Cullin-5 Interacting Protein. M. J. FAY, O. T. Meah, H. Nazeer, F. Farooqui, G. A. Karathanasis, and U. M. Shakur. Department of Pharmacology, Midwestern University, Downers Grove, IL 60515. Email: mfayxx@midwestern.edu

Cullin-5 (Cul5) has been implicated as a putative tumor suppressor in breast cancer since it is located on a region of chromosome 11(q22-23) that is associated with loss of heterozygosity. Previously we demonstrated a decrease in the expression of Cul5 mRNA in breast tumor tissue versus matched normal tissue, thus supporting a potential role for decreased expression of Cul5 in breast tumorigenesis. Cullin-5 is a member of the evolutionarily conserved Cullin protein family. The main cellular function attributed to Cullins is a role as scaffolding proteins within E3 ubiquitin ligase complexes. These E3 ubiquitin ligase complexes target various cellular proteins for ubiquitin-mediated degradation by the 26 S proteasome. Although Cullins are known to act as scaffolding proteins within E3 ubiquitin ligase complexes it is not clear what the cellular function(s) of Cul5 are with regard to tumorigenesis. To better understand the cellular functions of Cul5, a yeast two-hybrid screen using Invitrogen's Proquest Two-Hybrid System was implemented to identify Cul5 interacting proteins. The MaV203 yeast strain was co-transformed with bait construct (Cul5 ORF in frame with the GAL-4 DNA binding domain) and prey constructs (cDNA library in frame with the GAL-4 activation domain) and colonies containing putative Cul5 interacting proteins were identified by growth on plates lacking histidine. DNA sequencing and BLAST sequence similarity searching of the prey construct from several positive colonies revealed that alpha-actinin is a putative Cul5 interacting protein. Since alpha-actinin is a cytoskeletal protein involved with cell-cell and cell-extracellular matrix adherens junctions the interaction of Cul5 and alpha-actinin may affect cell motility and metastasis. Supported in part by NIH AREA grant CA85279.

VT-2003

CryoPlates[®] for Cytotoxicity Testing. L. H. CAMPBELL, K. A. Sarver, S. A. Miller, B. B. Leman, and K. G. M. Brockbank. Cell & Tissue Systems, Inc., 701 East Bay St., Charleston, SC 29403. Email: lcampbell@celltissuesystems.com

Cell-based screening assays provide the first check of the utility and cytotoxicity of given developmental compounds. Even with the development of more complex three dimensional tissue systems, the ease and high throughput of cell assays will always be a necessity. The only drawback to using cells for high throughput screening is the time and expense required to grow, maintain and plate cells for assays. We have developed a method for cryopreserving cells on microtiter plates. The utility of this system is that potentially any cell type can be preserved in a plate format so that upon thawing and recovery the cells are immediately ready for assessment. Development of this system has been performed using a bovine corneal endothelial cell line (BCE). This cell line was chosen because of its applicability to complement and potentially decrease the use of the Draize eye irritation test. Methods have been developed resulting in ~75% or better viability when BCE cells are cryopreserved in microtiter plates. While this appears to be a relatively simple task, many issues have had to be addressed, the greatest of which was achieving uniform viability and cell distribution across the plate after cryopreservation. This protocol is currently being refined for application with other cell types of interest. In conclusion, we have developed a convenient cryopreserved test system, based on a microtiter plate format that would supply cells ready for use in assays. It is anticipated that the availability of cell cultures in a bank will reduce both the costs and time required to screen potential new drugs.

VT-2004

Oxidative Control of Cell Signaling. B. L. UPHAM and James E. Trosko. Department of Pediatrics & Human Development, and National Food Safety & Toxicology Center, Michigan State University, East Lansing, MI 48824. Email: upham@msu.edu

For many years, research on oxidative stress focused primarily on determining how reactive oxygen species (ROS) damage cells by indiscriminate reactions with the macromolecular machinery of a cell, particularly lipids, proteins and DNA. However, many chronic diseases affiliated with oxidative stress, such as cancer, are not always a consequence of tissue necrosis, DNA mutations, or protein damage but rather to altered gene expression. Gene expression is highly regulated by the coordination of extra-, intra- and inter-cellular communication systems that will typically maintain tissue homeostasis by sustaining a balance between proliferation, differentiation and apoptosis. Therefore, much research has shifted to the understanding of how ROS can reversibly control the expression of genes at noncytotoxic doses through cell signaling mechanisms. Cell proliferation typically involves inhibition of gap junctional intercellular communication (GJIC) and the activation of mitogen activated protein kinase pathways (MAPK). We demonstrate that normal rat liver epithelial cells response to epidermal growth factor (EGF) will inhibit GJIC in addition to activating extracellular receptor kinase (ERK), a MAPK. However, inhibition of NADPH oxidase, which reduces oxygen to H₂O₂, with the very selective inhibitor, diphenyleneiodonium, prevented EGF from inhibiting GJIC suggesting that the generation of H₂O₂ is an essential component of the intracellular pathway controlling GJIC. Furthermore, we previously demonstrated that reduced-glutathione (GSH) was also a necessary cofactor of H₂O₂-induced inhibition of GJIC. These results demonstrate that ROS and GSH play essential roles in controlling EGF-dependent control of GJIC. Therefore, the overly simplistic approach of either preventing the generation of ROS or accelerate the removal by antioxidants could deleteriously alter normal signaling functions.

VT-2005

In Vitro Alternatives to In Vivo Ocular Toxicology Testing. C. J. HALLETT^{1,2}, J. G. Sivak^{1,2}, D. J. McCanna^{1,2,3}, V. L. Bantsev^{1,2}, and K. L. Moran^{1,2}. ¹University of Waterloo, School of Optometry, Waterloo, Ontario, CANADA, N2L 3G1 and ²University of Waterloo, Department of Biology, Waterloo, Ontario, CANADA, N2L 3G1; and ³Bausch & Lomb, Rochester, NY 14609. Email: cjhallet@uwaterloo.ca

It is widely believed that a battery of *in vitro* tests will be required to replace the current industry standard toxicology test, *in vivo* Draize test. Knowledge of the advantages and disadvantages of *in vitro* assays is essential to determine their potential for use in the proposal of an *in vitro* battery of tests. The Sodium Fluorescein Permeability Assay (SFPA), Agar Overlay Test (AOT), and Bovine Lens Assay (BLA) were explored using surfactants of known ocular toxicities: sodium dodecyl sulfate (SDS), benzalkonium chloride (BAK), Tween 20, and Triton X-100. The SFPA employs Madin-Darby Canine Kidney (MDCK) cells forming tight junctions within monolayers for treatment with test chemical. Treated monolayers were then washed with buffered saline prior to loading with sodium fluorescein. Sodium fluorescein that passed through the treated monolayer was measured via a fluorometer as an indirect measure of ocular toxicity. The AOT utilizes mouse fibroblast (L929) cells overlaid with agar-Minimum Essential Medium (MEM) and stained with 1.0% neutral red dye. Disks saturated with test chemical were placed on the agar-MEM layer, and following incubation, cytotoxicity is examined as zone of decolorization and percent of cell lysis. The BLA requires the lenses of abattoir-supplied bovine eyes to be isolated and cultured in supplemented M-199 culture medium. At 4 h, 8 h, and then in 24 h increments following test chemical treatment, the a laser beam is scanned through the lens in order to determine back vertex distance (BVD) and the standard error measurement (SEM) of the BVD as loss of optical quality of the lens is attributed to ocular toxicity of a test substance. Strengths and limitations of each assay were identified via this research including sensitivity level, objectivity level, the effects of filter use, and external factors. In addition, it was observed that neutral red dye (1.0%) underwent a chemical reaction when interacting with concentrations of SDS \geq 1%. The analysis of the above assays will be of great use when selecting tests to be included in a battery of tests proposed to replace the Draize test.

VT-2006

Comparative Cytotoxicity of Three In Vitro Viability Assays. ALANA RENAUD, Ann Wright, and Mary Mowrey-Mckee. CIBA Vision Corporation, A Novartis Company, Duluth, GA 30097. Email: alana.renaud@cibavision.novartis.com

PURPOSE: To compare three colorimetric *in vitro* assays using a murine fibroblast cell line (L929) by evaluating the cytotoxicity of benzalkonium chloride (BAK).

METHODS: Tests used were two cell viability assays using Alamar Blue (AB) and MTS/PES (MTS/PES), and a cell membrane integrity assay using neutral red (NRUR). L929 cells were plated onto 96 well plates, incubated to an 80% confluency and tested at 24 hours exposure to BAK solutions. BAK was diluted with growth media to yield 5, 2.5, 1.25, and 0.625 ppm solutions. The endpoint for the MTS/PES and NRUR assays was spectrophotometric measurement using a microplate reader. The endpoint for the AB assay was cytofluorometric measurement using a cytofluorometer. The MTS/PES and NRUR data compared the optical densities of test concentrations to the nontoxic optical densities of the control well and were expressed as a final percentage of viability. The AB data compared the fluorescence units of test concentrations to the nontoxic fluorescence units of the control well and were expressed as a final percentage of viability.

RESULTS: Cytotoxicity percentages were different between the three cell viability assays at BAK concentrations of 5, 2.5, and 1.25 ppm. MTS/PES, AB, and NRUR assays exhibited similar cytotoxicity percentage values for BAK at 0.625 ppm. Test concentrations were considered cytotoxic if percent viability versus control was less than 50%.

DISCUSSION: Percent viabilities observed using the MTS/PES, AB, and NRUR assays were different for BAK at concentrations of 5, 2.5, and 1.25 ppm. Low percent viabilities observed using the MTS/PES assay are partially attributed to the low level of succinate dehydrogenase present in the L929 cell line and its decreased potential for reduction of the dye. NRUR was the most sensitive assay with the most linear results. Cell line and metabolic properties of each cell line need to be considered when selecting a high throughput assay for cytotoxicity screening. AB and NRUR are useful for quantitative factor induced cytotoxicity screening within a 24 hour period of cell culture using L929 cells. Multiple assays can yield valuable information concerning *in vitro* cellular cytotoxicity.

VT-2007

A Non-animal Phototoxicity Test Using Epidermal Tissue Models and Cytokine Endpoints. G. DEGEORGE, A. Gilotti, M. Reeder, C. Kirk, T. Ripper, and D. Cerven. MB Research Laboratories, Spinnerstown, PA 18968. Email: mbinfo@mbresearch.com

The phototoxic potential of chemicals, cosmetics, consumer products and pharmaceuticals are of increasing concern to regulatory agencies and industry. Currently in the US, there are no regulatory agency-accepted *in vitro* assays or alternatives to costly, low-throughput animal phototoxicity tests. To address this need, we have developed and pre-validated a high-throughput *in vitro* screening test for phototoxicity, designated the Enhanced Phototoxicity Assay in Reconstituted Skin (EPARS). EPARS overcomes many of the limitations of the 3T3 NRU Viability test, which has been validated in Europe by ECVAM. Specifically: 1) EPARS is based upon a differentiated tissue model that closely parallels human skin morphology, instead of a fibroblast monolayer; 2) the tissues are composed of primary human keratinocytes, a more relevant model than a mouse tumor cell line; 3) test substances can be applied directly, avoiding the often problematic solubilization of formulations into culture media. In EPARS, the test substance is applied topically to the reconstituted human skin models, \pm UV irradiation. Phototoxic effects are determined by measuring MTT uptake of irradiated vs. non-irradiated tissues. In addition, to increase the sensitivity and specificity of the test, we measured the release of cytokines into the culture media via ELISA. Overall, EPARS proved to be an accurate and sensitive test for detecting phototoxic (photo-irritating) substances. PGE2 release was shown to be an early predictor of the toxic effects demonstrated in the viability assay. Release of IL-1 alpha, IL-1ra, IL-8 and TNF-alpha supported the results of the cell viability endpoint. Microarray analysis of gene expression showed that chlorpromazine treatment with UVA irradiation caused changes in gene expression that were not observed in untreated control tissues, tissues treated with UVA only, or chlorpromazine without UVA.

VT-2008

A High-throughput Unscheduled DNA Synthesis (UDS) Assay Using Flow Cytometry. M. KIRK, M. Reeder, T. Ripper, M. Fischl, D. Cerven, and G. DeGeorge. MB Research Laboratories, Spinnerstown, PA 18968. Email: mbinfo@mbresearch.com

There is a growing need in industry and regulatory agencies for an inexpensive screening assay that can reliably determine the genotoxic potential of the large number of new chemical compounds synthesized each year. The Unscheduled DNA synthesis (UDS) Assay is a proven method to identify and characterize genotoxic chemicals. It is based on the ability of damaged DNA to incorporate radiolabeled thymidine during the repair of various types of genetic lesions, which are subsequently detected by standard autoradiographic methods. However, several technical aspects of the standard UDS assay, including its use of radioactivity, have greatly limited its more widespread application. We have made modifications to the standard UDS assay through incorporation of BrdU incorporation and flow cytometry as a detection method. In addition, this is an *in vitro*, non-animal based test, using human hepatocyte cultures or avian embryonic hepatocytes, as well as less metabolically active human keratinocytes as the test system, therefore addressing all of the 3Rs of animal testing. We have also demonstrated the accuracy and sensitivity of this version of UDS, termed "FLUDS", which allows higher throughput and much shorter study times (a few days versus weeks for standard UDS assay). This new assay can discriminate between innate and biotransformed genotoxins, and is fully quantitative. It has reliably detected and characterized the UDS induced by ethylnitrosourea, aphidicolin, 2-acetylaminofluorene, and fluorene. Due to its high-throughput capability and other improvements over the standard UDS assay, the FLUDS assay now adds an important and humane tool of considerable value to regulatory agencies, academia as well as scientists in the pharmaceutical, chemical and consumer product industries.

VT-2009

Liver Tissue Engineering in Microgravity Environment. V. I. Khaoustov¹, D. Risin², N. R. Pellis², and B. Yoffe¹. ¹Baylor College of Medicine, Houston, TX 77030, and ²NASA, Biotechnology Program, Houston, TX 77058. Email: adimirk@bcm.tmc.edu

Background: The only available treatment for subjects with end-stage liver disease is liver transplantation. However, due to the shortage of donor livers, there is an urgent need to develop alternative approaches, including liver assist devices, hepatocyte transplantation and xenotransplantation. While these approaches require a readily available pool of primary hepatocytes, previous efforts have failed to establish long-term cultures of viable and differentiated hepatocytes. **Methods and Results:** Using a NASA-developed bioreactor we demonstrated that a simulated microgravity is conducive for maintaining long-term cultures of functional hepatocytes. We observed the formation of liver tissue-like structures from a suspension of single liver cells obtained by collagenase perfusion. Albumin mRNA was expressed throughout the 60-day culture and co-culture of hepatocytes with endothelial cells to stimulate albumin expression. Microsomal functions were demonstrated by activity of the p450 system. To optimize oxygenation and nutritional uptake within growing cellular aggregates, the cells were cultured in the presence of PGA scaffolds that resulted in improvement of liver remodeling. We found tissue-like structures comprised of hepatocytes, biliary epithelial cells and/or progenitor liver cells that were arranged as bile duct-like structures along nascent vascular sprouts and scaffolds. Electron microscopy revealed groups of cohesive hepatocytes surrounded by complex stromal structures and reticulin fibers, bile canaliculi, and tight junctions. Tissue-like structures were also examined for markers of apoptosis. We observed a decrease in ER stress following the reduction of mitochondrial membrane potentiality disruption, inhibition of Bip/GRP78 and caspase-12 activation. Subsequent studies demonstrated inhibited activation of effector caspases and apoptosis. Additionally we assessed the effect of simulated microgravity on the modulation of expression of a glucocorticoid receptor (GR), which is involved in termination of the stress response. Time-dependent decreases of GR mRNA levels were observed. This data is related to CPR critical questions for space flight risk 28: Human Performance Failure Due to Neurobehavioral Problems during space flight. Using cDNA microarray we demonstrated that simulated microgravity modifies the expression of hundreds of genes. **Conclusion:** A simulated microgravity is conducive for maintaining long-term cultures of hepatocytes. This cell culture model may assist in developing improved protocols for transplantation, gene therapy, liver assist devices, and facilitate studies of liver regeneration. Our results also suggest that microgravity has stimulating anti-apoptotic and anti-proliferative effects, and importantly, provide insight into mechanisms of microgravity induced neuro-behavioral problems during space flight.

VT-2010

Establishment and Characterization of 5 New Human Breast Cancer Cell Lines. J. L. KU, K. H. Kim, J. S. Choi, M. S. Jung, Y. K. Shin, I. J. Kim, K. J. Choi, S. K. Oh, D. Y. Noh, and J. G. Park. Laboratory of Cell Biology, Korean Cell Line Bank, Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-744, KOREA. Email: kujalok@cell.snu.ac.kr

Human breast cancer cell lines are difficult to establish in culture. Only about 20 cell lines are adequately characterized and widely used. We report the characteristics of five cell lines (designated, SNU-306, SNU-334, SNU-1528, SNU-1553 and SNU-1581), which were established from pathologically-proven three primary carcinomas and two pleural effusions obtained from five Korean breast carcinoma patients. Three cell lines grow as adherent cells, one cell line grows as floating aggregates and one line grows as both adherent and floating aggregates. All lines were free of mycoplasma or bacteria and were proven unique by DNA fingerprinting analysis (D1S1586 and D3S1765). An inframe deletion of 42 base pairs (14 codons) between codons 93 and 108 of p53 gene was found in SNU-1528. SNU-1528 cell line was highly resistant to paclitaxel (taxol). COX-2 was expressed in SNU-306 and SNU-1528 cell lines and estrogen receptor α (ER- α) was highly expressed in SNU-306 and low or undetectable in other cell lines. ER- β was expressed in SNU-1553 and SNU-1581 cell lines and progesterone receptor (PR) was expressed in SNU-306 cell line. E-cadherin gene was not expressed in SNU-1581 cell line. Since the establishment of breast cancer cell lines are difficult, we believe that these well-characterized 5 new cell lines from Korean breast cancer patients will be useful to study on biology of breast cancer.

VT-2011

Comparison of Alternative Toxicity Test Methods for Ocular Irritation Using Avian Chorioallantoic Membrane: HET-CAM vs. CAMVA. A. GILLOTI, G. DeGeorge, and D. Cerven. MB Research Laboratories, Spinnerstown, PA 18968. Email: mbinfo@mbresearch.com

Industry and some regulatory agencies are seeking alternatives to the Draize Eye Irritation Test in rabbit to address the three R's of decreased animal testing. Decades of research and validation have produced two non-animal-based ocular toxicity tests which use a keratinocyte based vascularized tissue as the test system; the CAM Vascular Assay (CAMVA) and the Hen's Egg Test-CAM (HET-CAM). The HET-CAM assay has been nominated by the EPA for evaluation as an alternative to the Draize test for ocular irritancy in rabbits. In the HET-CAM assay, test substances are applied topically to the CAM (at 10, 2.5 and 1% w/v). Ocular irritancy is predicted by calculating an irritation score (IS) based on the length of time until the exposure results in cell or tissue damage including hemorrhage, lysis or coagulation. This reaction time is determined by visual inspection of the CAM through the test chemical. Turbid test chemicals prevent the reaction time from being determined, and thus they cannot be evaluated in the HET-CAM assay. This presents a serious problem for determining the ocular irritancy potential of mixtures and cosmetic formulations. We routinely use the CAM Vascular Assay (CAMVA) to evaluate turbid or insoluble formulations of cosmetics and other products. In the CAMVA, the CAMs are exposed to various concentrations of a test substance for 30 minutes and vascular effects are monitored to determine a RC₅₀ for each test substance. We have used a 5-minute exposure time in the HET-CAM assay to determine the RC₅₀ of various test chemicals. HET-CAM derived RC₅₀ values were comparable to those determined in the CAMVA. In addition, the IS scores of translucent test substances were compared to the HET-CAM derived RC₅₀ values to develop a model for predicting ocular irritancy based on a concentration-dependent modification to the HET-CAM protocol.

E-2000

Effect of Green Tea and Garlic on Gastric Cancer Cells In Vitro Study. STEPHEN W. LI¹, Qiang Shen², and Xuejun FAN³. ¹Clear Brook High School, 4607 FM 2351 Friendswood, TX 77546; ²Breast Cancer Center, Baylor College of Medicine, Houston, TX 77030; and ³Department of Hybridoma Lab, Diagnostic Systems Laboratories, INC, 445 Medical Center Blvd., Webster, TX 77598. Email: jfan@dslabs.com

Millions of Americans suffer from peptic ulcer and gastric cancer. To combat these health problems, medical scientists have researched and discovered that *Helicobacter pylori* (*H. pylori*) are strongly associated with peptic ulcer and gastric cancer. Green tea contains many polyphenolic compounds (catechins), including the most important and dominant catechin, (-) Epigallocatechin Gallate (EGCG). Garlic contains organosulphur compounds, which are potent antioxidants. Therapeutic actions of green tea and garlic have been attributed primarily to its catechins and organosulphuric compounds, but more specific studies are needed to better clarify its pharmacological properties. Therefore, I have studied how green tea and garlic extracts kill the gastric cancer cells directly or indirectly by killing the *H. pylori* bacteria. Also, I examined how green tea and garlic extracts affects other types of cancer cells besides gastric cancer cells. In this project, I have designed three different experiments. First, the *H. pylori* were culturing together with different concentration of green tea (0.5, 5, 50 mg) and garlic (2, 20, 200 mg) extracts on blood agar plates for 3 days. Then, the gastric cancer cell lines (N87 and AGS), breast cancer cell lines (MCF-7 and T47D) and colon cancer cell line (CaCo2) were treated with different concentration of green tea (0.5, 5, 10 mg) and garlic (2, 20, 40 mg) extracts on 96-well plates for 4 and 8 hours. Then the amount of BrdU incorporated into live cells during DNA synthesis was measured by an ELISA reader. In order to prove my hypothesis, cell proliferation was evaluated by the cell counting after gastric cancer cell lines (N87 and AGS), breast cancer cell lines (MCF-7 and T47D) and colon cancer cell line (CaCo2) were cultured with different concentration of green tea (5, 25 mg) and garlic (20, 100 mg) extracts on 48-well plates for 4 hours. To demonstrate that the green tea and garlic extracts only kills cancer cells but not normal cells; a control experiment was performed. My peripheral mononuclear blood cells (PBMC) were culturing together with different concentration of green tea (5, 25 mg) and garlic (20, 100 mg) extracts on 48-well plates for 4 hours and then the number of live cells and dead cells were counted. The results illustrated that on blood agar plates showed that *H. pylori* growing were inhibited by green tea and garlic extracts at higher concentration. The results demonstrated that there were significant decreases in the DNA synthesis for all the cancer cell lines (above) after being treated with green tea and garlic extracts. For cell counting results, there were significant decreases in the number of live cells and increases in the number of dead cells for all the cancer cell lines (above) after being treated with green tea and garlic extracts. In the control experiment, the PBMC remained unaffected. In conclusion: the green tea and garlic extracts were extremely effective in killing *H. pylori*, and gastric cancer cells, breast cancer cells, colon cancer cells. My data strongly supports the idea that green tea and garlic extracts works as an anti-carcinogen and aid in prevention and treatment of gastric cancer cells as well as other cancer cells.