Presentation Abstracts:

Cell Surface/Attachment

Title: Polar surface attachment and biofilm formation of *Agrobacterium tumefaciens* requires contact-dependent extrusion of a unipolar polysaccharide analogous to the *Caulobacter* holdfast

Author(s): Elise R. Morton, Peter M. Merritt, Michael E. Hibbing, Thomas Danhorn, Cherie Blair and Clay Fuqua

Abstract: *Agrobacterium tumefaciens* is capable of forming dense biofilms on both abiotic and biotic surfaces, during saprophytic and parasitic growth. These large populations of bacteria may increase the overall efficiency of transformation while affording protection against host defense responses. Biofilm formation and infection of plant tissues are both dependent on the ability of cells to stably associate with a surface. Phosphorus-limitation, which is known to enhance expression of virulence genes, promotes biofilm formation, and enhances polar surface attachment. Staining of cells activated for the Pho regulon, with fluorescently-tagged wheat germ agglutinin (WGA), specific for N-acetyl glucosamine, revealed the presence of single fluorescent foci localized to one end of each labeled cell. This unipolar polysaccharide (UPP) is also present on attached cells grown in high phosphate, and is located on the pole of the cell adhered to the surface. A transposon mutation in Atu1236, homologous to the holdfast polysaccharide synthesis gene (hfsE) from *Caulobacter crescentus*, abolished biofilm formation, polar attachment and UPP synthesis. Atu1236 is located within a cluster that encodes several putative polysaccharide biosynthetic functions, and is conserved and syntenous among many rhizobia. Expression of the UPP is absolutely required for A. *tumefaciens* polar attachment to abiotic surfaces and binding to Arabidopsis root tissues. A plasmid-borne copy of Atu1236, complements the mutant phenotypes, and significantly enhances adherence when expressed in the wild type background. Extrusion of the UPP is coincident with surface contact and this is not prevented by antibiotics that block protein synthesis such as tetracycline and kanamycin.

Title: Lon Protease and a Type VI Secretion System Regulate Surface Attachment in *Agrobacterium tumefaciens*

Author(s): Lois Banta[1], David Rogawski[1], Ian Buchanan[1], Jason Fan[1], Gape Machao[1], Shengchang Su[2], Stephen Farrand[2], Amelia Tomlinson[3], Clay Fuqua[3], Yuan Ze-Chun[4], Jen Strater[5], Brad Goodner[5], and Gene Nester[4]

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Abstract: Polar attachment and biofilm formation are two manifestations of *Agrobacterium tumefaciens* interactions with host cell surfaces. The polar localization of several components of the T-DNA/protein translocation machinery and of the *virB*-encoded T-pilus itself suggest that one or more of the VirB proteins might contribute to polar attachment. Indeed, scanning electron microscopy revealed that in wild-type strain A348, pTi-encoded functions and in particular the *virB* genes, stabilized bacterial association with Arabidopsis leaves, but were not absolutely essential for polar interactions. Cells lacking the Lon protease exhibited defects specifically in VirB-mediated transport and failed to elaborate an extracellular T-pilus. Significantly, however, the lon mutant was not deficient for host-cell attachment; in fact it was markedly hyperadhesive on both leaves and roots. The pronounced enhancement in polar attachment by the lon mutant, coupled with a substantial rise in Lon protein accumulation that coincides with *vir* gene induction, suggest that Lon may mediate a switch from a preliminary polar interaction to a T-pilus-stabilized association; we propose that in the lon mutant, this progression is stalled at the initial stage of plant host attachment, resulting in a failure to properly up-regulate *virB* gene expression. A second protein implicated in the Lon-dependent shift to virB transcription is Ros, a regulatory protein previously identified as a repressor of the virC/D operons and a positive regulator of exopolysaccharide production. We have found that Ros also represses the imp operon, which encodes a putative Type VI secretion system (T6SS). The recently identified T6SS gene clusters contribute to host cell interactions in a variety of gram-negative pathogens and symbionts. In *Agrobacterium*, T6SS genes are found in strains C58 and S4, but not K84 or A4. Mutants lacking one or more imp operon genes exhibited complex, bacterial cell-density dependent, patterns of altered virulence on both tobacco and *Arabidopsis*. Most intriguing, we have discovered that bacterial adhesion to abiotic surfaces is dramatically enhanced in the imp mutants. Microscopic analysis of flow cell biofilms demonstrated that complete deletion of the imp operon results in an increased number of microcolonies which have strikingly greater biomass relative to those from wild type. Although a T6SS in *Pseudomonas aeruginosa* has previously been shown to be co-regulated with biofilm formation and exopolysaccharide production, this is to our knowledge the first indication that a T6SS is directly involved in biofilm formation.

Title: The ExoR protein of *Agrobacterium tumefaciens* is a novel global regulator that controls diverse functions influencing motility, exopolysaccharide synthesis and biofilm formation

Author(s): Amelia D. Tomlinson[1], David Rogawski[2], Lois M. Banta[2] and Clay Fuqua [1]

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Abstract: *Agrobacterium tumefaciens* C58 forms biofilms on abiotic surfaces and plant tissues. Mutation of exoR results in derivatives that cannot attach efficiently to either type of surface. As in *Sinorhizobium meliloti*, the exoR mutation results in overproduction of the exopolysaccharide succinoglycan (SCG) and elevated expression of SCG biosynthetic (exo) genes. SCG is not required for biofilm formation by *A. tumefaciens*. Overproduction of SCG in the exoR mutant is not responsible for the abiotic biofilm deficiency, but does appear to prevent attachment to plant tissues. The exoR mutant also manifests reduced motility, with fewer flagella and less flagellin than wild type. The ExoR protein is not a typical transcriptional regulator, but rather contains an N-terminal secretion signal, and several tetratricopeptide repeats (TPRs), domains thought to mediate protein-protein interactions. ExoR is conserved among several alpha-proteobacteria, including animal pathogens, and free-living bacteria. We hypothesize that ExoR must interact, at some level, with a transcription regulation system to control SCG biosynthesis and exo gene expression. In contrast to *S. meliloti* ExoR does not function through the ChvG-ChvI two-component system. Microarray analysis of the ExoR transcriptome revealed a large network of regulated genes, some activated and some repressed by ExoR. This work has provided striking correlation between the mutant phenotypes observed and target genes, indicating negative regulation of exo genes and positive control of motility/chemotaxis-related genes. In addition, a host of cellular functions are impacted by ExoR, most notably including repression of the imp genes, encoding a Type VI secretion system that limits biofilm formation.

Plant Response to *Agrobacterium* Infection-Session I

Title: Regulation of *Agrobacterium vitis* induced plant responses and swarming motility

Author(s): T. J. Burr, L. Cursino-Parent, D. Zheng, G. Hao and S. Sule

Abstract: Four members of the LuxR family of transcriptional regulators (*aviR*, *avhR*, *avsR* and *avxR*) and one acyl-homoserine lactone synthase, *avsI*, are involved in quorum-sensing regulation in *Agrobacterium vitis*. All of these genes are essential for expression of a hypersensitive response on tobacco and for necrosis of grape tissues. In contrast, a subset of the genes is associated with the ability of *A. vitis* to produce a swarming phenotype on half-strength potato dextrose agar. AvsR regulates expression of *avsI* and also *avxR*. The ability of *A. vitis* to express a swarming motility phenotype is first visualized by diffusion of a surfactant-like material from bacterial cells spotted on conducive medium within a few minutes of spotting. All *A. vitis* strains tested were able to swarm whereas none of the *A. tumefaciens* or *A. rhizogenes* strains developed this phenotype. *aviR*, *avsR* , *avxR* and *asvI* mutants did not show the surfactant diffusion characteristic and were swarming negative. Complemented aviR regained swarming activity. In addition the *avsI* mutant was complemented by growing adjacent to *Agrobacterium* strain NT1 carrying *avsI* and producing long chain AHLs. The biological significance of swarming to bacterial survival and plant interactions is being investigated.

Title: Response of Plants to Infection and Transformation with *Agrobacterium tumefaciens*

Author(s): Marina Efetova, Rainer Hedrich, Rosalia Deeken

Abstract: The transfer of the T-DNA from virulent *Agrobacterium tumefaciens* strains into the plant genome is one of the most extreme triggers which substantially alter the cell fate of higher plants. Our studies have focused on physiological changes and adaptations of the host to the infection and transformation with agrobacteria. The profiles of metabolites and signaling molecules were compared with alterations in genome-wide gene expression changes. Three different stages of infection were analyzed: (i) before (3 hours post infection) and (ii) after T-DNA-integration (6 days post infection) as well as (iii) of fully developed crown galls (30 - 35 days post infection). For these studies the basis of inflorescence stalks of Arabidopsis thaliana was infected with the oncogenic *Agrobacterium tumefaciens* strain C58 or with a T-DNA-deleted non-oncogenic strain (GV3101). In addition *Arabidopsis* mutants with altered abiotic stress responses or defense reactions were used to study their impact on tumor development. Our data indicate that at early time points of infection the pathogen defense reactions are suppressed until the T-DNA is integrated. After T-DNA integration pathogen defense reactions are activated in the host plant and the fully developed tumor initiates drought stress protection mechanisms. At this stage of infection the crown gall development can no be longer be prevented but the degree of its growth can be restricted. On the meeting we will present our recent findings concerning the host responses to the T-DNA integration event.

Title: Salicylic acid, γ-amino butyric acid, and indoleacetic acid influence *Agrobacterium tumefaciens* through independent but overlapping signaling processes

Author(s): Ze-Chun Yuan, Denis Faure, and Eugene W. Nester

Abstract: Signaling in rhizosphere has profound effects on the physiology and function of both plant and bacterial species. DNA transfer into plant cells by *Agrobacterium* requires the bacterial virulence genes (vir regulon) to be induced by several signals in the rhizosphere, including an acidic environment (pH around 5.5), plant secreted sugars, as well as plant derived phenolic compounds. In addition, *Agrobacterium* can increase its community infectivity by transferring its tumor-inducing plasmid (Ti plasmid) to other *Agrobacterium* cells lacking the plasmid. This conjugal transfer is tightly regulated by the level of the quorum-sensing signal, acyl-homoserine lactone (acyl-HSL). Previous studies by Chevrot et al. found that the plant-derived compound -amino butyric acid (GABA) activates *Agrobacterium* lactonase (AttM) which can degrade acyl-HSL#1. In addition, previous studies from our lab revealed that plant signal indoleacetic acid (IAA) inhibits expression of the *Agrobacterium vir* regulon#2. Our recent studies have demonstrated that salicylic acid, a key signal molecule in regulating plant defense in response to a wide variety of pathogens, at physiologically relevant levels, inhibits *Agrobacterium* virulence by interfering with signal transduction between the sensor kinase VirA and the response regulator VirG. We also demonstrated that salicylic acid, like GABA, can modulate *Agrobacterium* quorum-sensing by activating the expression of AttM, thereby resulting in degradation of acyl-HSL#3. Using microarray and genetic analyses, we obtained transcriptome profiles of *Agrobacterium* in response to GABA, salicylic acid, and IAA. Our results indicate that these three plant derived signal molecules affect *Agrobacterium* by modulating independent as well as overlapping signal pathways. Furthermore, our data reveal that the activation of acyl-H S L degradation and the inhibition of *vir* regulon expression are independent processes (Yuan ZC, Faure D, and Nester EW. Manuscript in preparation). References: #1. Chevrot R, Rosen R, Haudecoeur E, Cirou A, Shelp BJ, Ron E, Faure D. GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. PNAS. 2006. 9;103(19):7460-4. #2. Liu P, Nester EW. Indoleacetic acid, a product of transferred DNA, inhibits *vir* gene expression and growth of *Agrobacterium tumefaciens* C58. PNAS. 2006. 21;103(12):4658-62. #3. Yuan ZC, Edlind MP, Liu P, Saenkham P, Banta LM, Wise AA, Ronzone E, Binns AN, Kerr K, Nester EW. The plant signal salicylic acid shuts down expression of the *vir* regulon and activates quormone-quenching genes in *Agrobacterium*. PNAS. 2007. 10;104(28):11790-5.

Plant Response to *Agrobacterium* Infection-Session II

Title: Characterization of *Arabidopsis* mutants that are hyper-susceptible to *Agrobacterium*-mediated transformation (*hat* mutants)

Author(s): Nagesh Sardesai, Huabang Chen, Joerg Spantzel, and Stanton B. Gelvin

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Abstract: We used activation tagging to identify *Arabidopsis* mutants that are hyper-susceptible to *Agrobacterium* transformation. We identified eight such hat mutants, and isolated T-DNA/plant junctions from five of them. The *hat1* mutant contains a T-DNA insertion in the 7th exon of a gene encoding a cellulose synthase-like (CSL) protein. RT-PCR indicated that CSL was down-regulated whereas a neighboring gene, UDP-glucosyl transferase (UGT), was strongly up-regulated. Over-expressing a UGT cDNA in wild-type plants resulted in increased root transformation. Knockdown mutants of CSL also showed a mild hat phenotype. The hat3 mutant has a T-DNA insertion in the 5' untranslated region of a myb transcription factor (MTF). RT-PCR analyses revealed low MTF transcript levels. Three MTF T-DNA insertion mutants showed increased transformation but MTF over-expressing plants did not show decreased transformation. We are currently conducting microarray analysis of wild-type, MTF over-expressing, and MTF knockdown plants to identify genes regulated by MTF. Two independent mutants, *hat*4 and *hat7*, have T-DNA insertions in the 5' region of a potassium transporter family (PTF) gene but did not show alterations in PTF transcripts. None of the genes in the proximity of this T-DNA showed differential regulation. The hat5 mutant has a T-DNA insertion in the intergenic region between a thioredoxin gene and an integral membrane transporter family (IMTF) gene, with high levels of transcript accumulation for both genes. Thioredoxin over-expressing plants did not recapitulate the hat phenotype. We are currently generating IMTF over-expressing transgenic plants to assess the importance of this gene in transformation.

Title: ***Agrobacterium*** **attachment to** ***Arabidopsis*** **roots induces the systemic expression of a defense-response gene, PAL1**

Author(s): Nagesh Sardesai, Veena, Clay Fuqua, and Stanton B. Gelvin

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Abstract: *Agrobacterium*-mediated transformation is a result of a complex interaction between the bacterium and the host plant. However, little information is available about the factors responsible for biofilm formation on the plant root surface, and defense responses mounted by the plant against the perceived pathogen attack. The formation of biofilms and host defense responses may be linked. The Arabidopsis defense-response gene phenylalanine ammonia lyase (PAL1) is up-regulated in response to infection by *Agrobacterium*. We fused the PAL1 promoter with EYFP and generated transgenic Arabidopsis containing this transgene. PPAL-EYFP plants expressed the PAL1 promoter in the root vasculature. We used mCherry-tagged wild-type and mutant *A. tumefaciens* strains to investigate interactions between the bacteria and the plant. *A. tumefaciens* C58 could form biofilms on *Arabidopsis* roots by 6 hours after inoculation. When PPAL-EYFP plants were infected with high concentrations of *A. tumefaciens*, the PAL1 promoter expressed in root epidermal cells at different times depending on which *Agrobacterium* strain was used. An *A. tumefaciens* strain over-expressing the SinR transcription factor induced PPAL-EYFP expression in root epidermal cells at colonization sites as early as two hours. A motA *A. tumefaciens* mutant induced a systemic response in leaves 24 hours after infection. This was the only tested strain that induced a systemic response. We are currently investigating the extent and kinetics of PAL1 promoter response to *Agrobacterium* in various *Arabidopsis* mutants altered in their defense responses.

Title: The SCF (SKP1/CUL/F-BOX) ubiquitin ligase complex plays an important role in T-DNA transfer and integration

Author(s): Ajith Anand and Kirankumar S. Mysore

Abstract: Genetic transformation of plants by *Agrobacterium tumefaciens* involves transfer and integration of the bacterial T-DNA (transferred DNA) into the plant genome. It is speculated that the T-DNA is thought to be imported into the plant nucleus as a DNA-protein complex (T-complex). The essential components of the T-complex include the single stranded T-DNA, virulence proteins (VirD2, VirE2, VirE3 and VirF) and host proteins. This T-complex has to be uncoated of its cognate protein before integration, which is probably achieved by targeted proteolysis mediated by VirF and the ubiquitin proteosome complex (UPS). Protein stability and degradation through UPS is an important mechanism that underlies many cellular processes in eukaryotes. The polyubiquitinization pathway involves several classes of enzymes, the most interesting being the ubiquitin protein ligases (or E3). The E3s fall in different families, among which SCF (SKP1-CUL1-F-box) is the largest and best characterized. Based on a reverse genetics approach using virus-induced gene silencing (VIGS) and through identification of *Arabidopsis* mutants of the SCF complex, we demonstrate that skp1 (suppressor of kinetochore protein 1) and sgt1 (suppressor of the G2 allele of Skp1) are critical to plant transformation. Using stable transformation assays and MUG assays we observed that the SGT1 and Skp1 gene silenced plants and the skp1 and sgt1 mutants are recalcitrant to transformation. MUG assays further suggested the skp1 and sgt1 mutants are deficient in transient transformation. However, the skp1 and sgt1mutants are amenable to transformation by either micro-bombardment or germ-line transformation. Based on these findings we suggest that the SCF complex plays a critical role in the T-DNA transfer and integration.

Chromosome Biology and T-DNA Integration

Title: Over-expression of *Arabidopsis* chromatin genes results in increased transformation efficiency and/or transgene expression

Author(s): Gabriela N. Tenea, Joerg Spantzel, Lan-Ying Lee, Susan Jonhson, Yanmin Zhou, Heiko Oltmanns and Stanton B. Gelvin

Abstract: Previous work from our laboratory indicated an essential role for *Arabidopsis* histone genes in T-DNA integration. RNAi targeted against 109 *Arabidopsis* chromatin genes further demonstrated a role for other chromatin proteins, such as SGA1, in *Agrobacterium*-mediated transformation. We investigated the effects of over-expressing numerous *Arabidopsis* histone cDNAs and an anti-silencing factor A (SGA1) cDNA on transformation and transgene expression. Transgenic *Arabidopsis* plants containing additional copies of cDNAs encoding histone H2A (HTA), histone H4 (HFO), or SGA1 displayed increased susceptibility to transformation. Over-expression of all tested histone H2B (HTB) and most histone H3 (HTR) cDNAs did not increase transformation. A parallel increase in transient gene expression was observed when the histone HTA or HFO cDNAs were co-transfected, together with a plant active *gusA* gene, into tobacco protoplasts. Using RT-PCR, we also detected an increase in *gusA* transcripts when the histone HTA1 cDNA was over-expressed in protoplasts. No such increase in *gusA* activity was seen when a SGA1 cDNA was co-transfected with a *gusA* gene into BY-2 protoplasts. These results suggest that over-expression of HTA and HFO increases transformation by stimulating transgene expression, whereas over-expression of SGA1 increases transformation by a different mechanism. Over-expression of histone or SGA1 cDNAs does not increase expression of a previously integrated transgene, nor could HTA1 reverse silencing. These data suggest that histones may increase transgene expression by working directly on the promoter of incoming DNA, or that histones may play a role in stabilizing transgene DNA (and thereby transgene expression) during the initial stages of transformation.

Title: ***In vitro*** **interactions between plant nucleosomes and VIP1 or reconstituted T-complex**

Author(s): Benoît Lacroix, and Vitaly Citovsky

Abstract: In the last steps of plant genetic transformation by *Agrobacterium tumefaciens*, which lead to integration of the T-DNA into the host genome, interactions between the T-complex (T-DNA and its associated bacterial and host proteins) and chromatin are most likely required. The role of core histones is central in this process, and it was shown that VIP1 (VirE2 interacting protein 1) does bind to the different core histones. We have investigated *in vitro* interactions between VIP1, as well as other components of the T-complex, and purified plant nucleosomes. In the system we have developed for this study, purified mononucleosomes from cauliflower florets were linked onto 96 well-plates, and binding of the tested proteins to immobilized nucleosomes was assessed by a modified ELISA protocol. VIP1 showed strong and specific interaction with nucleosome-embedded histones, even at a high stringency. Whereas VirE2 was able to bind to nucleosomes very weakly, and when low stringency washes were applied, a significant association between VirE2 and nucleosomes was observed only in presence of VIP1, showing that VIP1 is able to bridge the interaction between chromatin and VirE2. Moreover, the presence of VIP1 was also necessary to mediate binding of the VirE2-ssDNA complex to nucleosomes. We are currently exploring the influence of covalent histone modifications on nucleosome-VIP1 and nucleosome-reconstituted T-complex interactions, as well as the possible role of other factors known to interact with the T-complex in modifying these molecular associations.

Title: Identification and Characterization of DNA Repair Components Involved in *Agrobacterium*-Mediated Plant Transformation

Author(s): ZARIR VAGHCHHIPAWALA AND KIRANKUMAR S. MYSORE

Abstract: Little is known about the plant components involved in the T-DNA integration process during *Agrobacterium*-mediated plant transformation. It is speculated that T-DNA integration occurs via a non-homologous recombination pathway involving the non-homologous end joining (NHEJ) machinery. To identify plant genes involved in the T-DNA integration process, we used a virus-induced gene silencing-based reverse genetics approach. From an initial screen of 10 genes that play a role in DNA repair, four genes seemed to be involved in *Agrobacterium*-mediated plant transformation. We further characterized one of these genes, XPB1 (Xeroderma Pigmentosum complementation group B, a RAD25 homolog). XPB1 is part of the TFIIH complex, which is integral in the nucleotide excision repair pathway (NER). Silencing of XPB1 in *N. benthamiana* lead to a significantly reduced stable transformation efficiency. Arabidopsis thaliana xpb1 mutant was recalcitrant to *Agrobacterium*-mediated stable tranformation but not for transient tranformation. We propose that in addition to NHEJ pathway, NER pathway also plays a role in T-DNA integration. We also studied the role of other known members (from mammalian studies) of the NHEJ pathway in *Agrobacterium*-mediated plant transformation using *Arabidopsis* mutants and the preliminary results will be presented.

Title: Blocking T-strand conversion to double-stranded intermediates by expression of single-stranded DNA binding proteins

Author(s): Mery Dafny-Yelin, Raz Dafny, Lorenzo Prieto, Avner Levy and Tzvi Tzfira

Abstract: *Agrobacterium* delivers its T-strand into the host-cell nucleus where it can be converted into double-stranded molecules. Various studies have revealed that double-stranded (ds) T-DNA intermediates can serve as substrates by an as yet uncharacterized integration machinery. Nevertheless, the possibility that T-strands can still be the substrates for integration cannot be excluded. To further investigate the route taken by T-DNA molecules on their way to integration, we attempted to block the conversion of T-strands to double-stranded intermediates prior to integration. We produced transgenic plants which overexpressed three protein subunits of DNA Replication Factor-A (RFA) from yeast. These subunits, RFA1, RFA2 and RFA3, function as a complex in yeast cells which is capable of binding to single-stranded (ss) DNA molecules, promoting the repair of double-strand breaks. RFA overexpression in *Nicotiana bentamiana*, however, resulted in increased sensitivity to DNA-damaging agents relative to wild-type plants, indicating that heterologous and high expression of RFA may interfere with the host DNA-repair machinery, most likely by interacting with exposed DNA strands at genomic break sites. RFA-transgenic plants were defective in T-DNA expression, as determined by infection with *Agrobacterium* cells carrying the GUS intron reporter gene. Gene expression was not blocked when the reporter gene was delivered into plant cells by microbombardment. Confocal microscopy analysis revealed that yeast RFA forms a complex which resides within the plant nuclei and preliminary data showed that the cell-to-cell movement of the ssDNA bean dwarf mosaic virus is defective in RFA plants. These observations suggest that RFA may interfere with the T-strand's conversion to its double-stranded form, and by implication, block its expression and integration into the host genome.

Agrobacterium Technologies

Title: A New Broad Host Range Vector for Very Tightly Controlled Expression of Cloned Genes in the Alpha Proteobacteria Including *Agrobacterium* and *Brucella*

Author(s): Stephen K. Farrand[1], R. Martin Roop, II[2], and Sharik R. Khan[1]

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Abstract: In our studies on TraR, the quorum-sensing activator of the Ti plasmid conjugative transfer system, we had need of a vector in which the gene could be tightly repressed and induced rapidly. Current expression vectors, built on *lac* or *ara* regulatory components exhibited unacceptably high basal levels of expression of our cloned *traR* gene under repressing conditions. To deal with this problem we considered how LacI regulates the *lac* operon of *E. coli*. Maximum repression requires cooperative interaction of LacI tetramers at O_1 in the promoter region and one of two other operators, either O_2 located in *lacZ*, or O_3 located in *lacI*. Vectors based on P_{lac} lack O_2 and the *lacI* gene, when present, often is inserted as an afterthought. Consequently, the *lacI* gene is not positioned to allow for efficient interaction of LacI with O_1 and O_3. We reconstructed a derivative of the broad-host-range plasmid pBBR1MCS-5 such that an interval from the upstream UTR of $lacI^q$ through the *lac* promoter-O_1 complex and ending 3′ to *lacZ*α is virtually identical to the wild-type configuration. The only relevant modifications are the substitution of the mcs from pBBR1MCS with that from pBluescript and an *Nde*I site engineered at the ATG of *lacZ*α. A second version of this vector was constructed from pBBR1MCS-2 generating pSRKKm that confers resistance to kanamycin. A derivative of pSRKGm in which *uidA* was cloned as an *Nde*I fusion was tested for its regulatory properties in *A. tumefaciens* C58. In the absence of IPTG, levels of GUS activity were not significantly higher than background while addition of the inducer yielded a 15-20-fold increase in activity of the reporter. Using the *lacZ*α of the native pSRKGm vector, similar patterns of regulation of expression were detected in *E. coli* DH5α under uninduced and induced conditions. In strain C58, addition of IPTG resulted in induction of measurable levels of GUS activity within the first 30 minutes, and high levels of activity for as long as 20 hours after addition of inducer. We also tested pSRK-based *uidA* reporters in four other α-proteobacteria, *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, *Caulobacter crescentus*, and *Brucella abortus*, and one other γ-proteobacterium, *Pseudomonas fluorescens*. In all cases uninduced levels of GUS activity were not significantly above background. In *C. crescentus* addition of IPTG resulted in a 60-fold increase in GUS activity while induction levels ranged from 15- to 100-fold in the other tested bacteria. Derivatives of C58 in which we introduced *traR* cloned in pSRKGm failed to transfer their Ti plasmids at detectable frequencies in the absence of induction. Addition of IPTG to such donor cultures resulted in the strong induction of plasmid transfer. The vectors replicate stably at low copy number, are compatible with Ti plasmids and with IncP and IncQ vectors, and do not detectably influence traits of interest in *Agrobacterium*. Genes can be cloned into the mcs or at the ATG of *lacZ*α using the unique *Nde*I site, all of which disrupt *lacZ*α allowing blue-white screening in appropriate *E. coli* hosts.

Title: Peptide aptamers for defining protein function

Author(s): Stanton B. Gelvin, Zhuzhu Zhang, and Lan-Ying Lee

Abstract: Peptide aptamers (from the Latin aptus for "fitting") are short peptides of random sequence that can interact with specific target proteins in vivo. As commonly used, these peptides are generally 15-20 amino acids-long. This length provides enough flexibility for the peptide to assume various conformations while reducing the probability of randomly creating a stop codon in the aptamer coding sequence. We are currently developing peptide aptamer "mutagenesis" technology for use in plants. As proof of concept, we are targeting VirE2 with various 20-mers from the VirE2 protein sequence. VirE2 is known to interact with VirE1, VirE2, importin a, and VIP1; for many of these protein pairs, sites important for interaction with VirE2 are known. We have designed an aptamer expression cassette, based upon pSAT vectors, which uses bimolecular fluorescence complementation to detect aptamer-protein interactions. The cassette consists of a CaMV double 35S promoter + full-length mCherry + a multiple cloning site for insertion of aptamer coding sequences + nVenus + CaMV polyA addition signal. We have individually tagged VirE2 with cCFP and nCerulean. Dimerization of VirE2 brings together cCFP and nCerulean, generating blue fluorescence. Interaction of VirE2-nCerulean with the mCherry-aptamer-nVenus polyprotein generates yellow fluorescence. Expression of the aptamer cassette results in red mCherry fluorescence. Interaction of the aptamer with VirE2 may inhibit VirE2 function, resulting in decreased transformation of plant cells expressing the aptamer. We are testing these constructions in tobacco BY-2 cells and transgenic *Arabidopsis*. Preliminary results indicate that multiple aptamer polyproteins can interact with VirE2 in tobacco cells.

Title: Stable Recombinase Mediated Cassette Exchange in *Arabidopsis*

Author(s): Jeanine Louwerse, Miranda van Lier, Dirk van der Steen, Clementine de Vlaam, Paul Hooykaas and Annette Vergunst

Abstract: Site-specific integration is an attractive method for the improvement of current transformation technologies aimed at the production of stable transgenic plants. We used Cre-lox technology to obtain directed integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* using a method called Recombinase-Mediated Cassette Exchange (RMCE). The use of site-specific recombination systems for directed integration requires a two-step procedure: We first created a target plant line containing a recombination site, which was subsequently used as a landing platform for integration of T-DNA delivered in a second round of transformation. In RMCE, both the genomic and T-DNA replacement cassette are flanked by two heterospecific lox sites, which are incompatible with each other to prevent unwanted cassette deletion. The Cre recombinase was delivered on a co-transforming T-DNA. We effectively exchanged the coding region of a loxP/lox5171-flanked bialaphos resistance (bar) gene for a loxP/lox5171-flanked T-DNA replacement cassette containing the neomycin phosphotransferase (*nptII*) coding region. Here, we will present our results, and discuss the applicability of this *Agrobacterium*-based RMCE strategy in comparison with other targeting strategies.

Title: T-DNA trapping by genome-wide double-strand DNA breaks

Author(s): Amanda S. Freed and Tzvi Tzfira

Abstract: Although several different cellular pathways have been suggested to describe T-DNA's integration into plant cells, the mechanism by which T-DNA molecules integrate into the host genome is still largely unknown. Recent reports have revealed the roles played by DNA repair and maintenance proteins during the integration process. More specifically, the critical roles played by KU80, a nonhomologous end-joining host protein, and genomic double-strand breaks (DSBs) in T-DNA integration have led to the notion that T-DNA molecules can integrate as double-stranded intermediates. To deepen our understanding of the integration of T-DNA molecules into various genomic locations, a method for site-specific induction of DSBs was needed. We reasoned that transient expression of a restriction enzyme, capable of producing multiple genomic DSBs, would be useful for analyzing T-DNA integration into multiple genomic locations. To this end, we harnessed the power of AscI, an 8-base restriction enzyme capable of recognizing ca. 80 sites in the Arabidopsis genome. Proper engineering of this prokaryotic protein enabled its expression, translocation into the host cell nucleus and digestion of both extrachromosomal and genomic DNA. More importantly, coupling the expression of AscI with *Agrobacterium* infection resulted in the trapping of T-DNA molecules into AscI recognition sites in the *Arabidopsis* genome. Using a collection of primers which were designed to produce a unique amplification pattern for all genomic AscI recognition sites, we were able to monitor and detect the occurrence of DSB-site disruption and/or site-specific T-DNA integration events. We generated nearly a thousand independent T-DNA integration events and discovered that they were equally distributed throughout the *Arabidopsis* genome, regardless of AscI-site locations. We are currently plotting our D S B-mediated T-DNA integration distribution map against random T-DNA integration events, as mapped by the SALK T-DNA collection, and analyzing the statistical significance of our data in order to provide a model for DSB-mediated T-DNA integration.

Title: Design of an industrial *Agrobacterium tumefaciens* strain for use with magnifection.

Author(s): Sylvestre Marillonnet

Abstract: The magnifection process consists of infecting entire *N. benthamiana* plants by systemic delivery of viral vectors using *Agrobacterium tumefaciens*. The viral vectors used at present with magnifection consist of TMV-based vectors that are able to move in infected plants from cell to cell but are unable to move systemically. In order to infect entire plants, the plants are inverted and dipped in a solution of *Agrobacterium*, and are vacuum-infiltrated. One potentially limiting factor for large-scale industrial applications will be the necessity of growing and handling large amounts of *Agrobacterium*. Fortunately, the use of viral vectors engineered for efficient expression allows using a 1000 fold-diluted *Agrobacterium* suspension (related to the concentration of agrobacterial cells in the growth medium) and still obtain efficient infection of infiltrated leaves. Although all handling of agrobacteria will be performed in a contained environment, we are now working on engineering an *Agrobacterium* strain that will have reduced growth and T-DNA transfer ability outside the production facility, in case of unintended release in the environment.

Virulence and Secretion Systems

Title: Secretome analysis uncovers an Hcp-family protein secreted via a type VI secretion system in *Agrobacterium tumefaciens*

Author(s): Hung-Yi Wu, Pei-Che Chung, Hsiao-Wei Shih, Sy-Ray Wen, and Erh-Min Lai

Abstract: *Agrobacterium tumefaciens* is a plant pathogenic bacterium capable of secreting several virulence factors into extracellular space or the host cell. In this study, we used shotgun proteomics analysis to investigate the secretome *of A. tumefaciens*, which resulted in the identification of 12 proteins, including two known and 10 potential secretory proteins. Interestingly, all identified proteins are known or putative periplasmic or membrane proteins except for one unknown protein named hemolysin-coregulated protein (Hcp), a predicted soluble protein without a recognizable N-terminal signal peptide. Western blot analysis revealed that *A. tumefaciens* Hcp is constitutively expressed and secreted when grown in both minimal and rich media. Further biochemical and immunoelectron microscopy demonstrated that intracellular Hcp is mainly localized in the cytosol, with a small portion in the membrane system. To investigate the secretion mechanism of Hcp in *A. tumefaciens*, we generated mutants with deletions of a conserved gene, *icmF*, or the entire putative operon encoding a recently identified type VI secretion system (T6SS). Western blot analysis indicated that Hcp was expressed but not secreted into the culture medium in mutants carrying deletions of *icmF* or *t6ss*. The secretion deficiency of Hcp in *icmF* mutant was complemented by heterologous expression of *icmF* in trans, suggesting that *icmF* is required for Hcp secretion. The deletion of *hcp* results in 20-25% reduction of tumorigenesis efficiency while no significant difference can be observed in deletions of *icmF* or *t6ss* in comparison to wild type by tumor assays on potato tuber discs. This work provides supporting evidence on this conserved protein secretion system used by both plant and animal pathogenic bacteria for intimate interactions with their hosts.

Title: The *Agrobacterium* 6b protein has histone-chaperone-like activity

Author(s): Yasunori Machida, Shinji Terakura, Yoshihisa Ueno, Chiyoko Machida

Abstract: Protein 6b, encoded by T-DNA from the pathogen *Agrobacterium*, stimulates the plant-hormone-independent division of cells in culture in vitro and induces aberrant cell growth and the ectopic expression of various genes, including genes related to cell division and meristem-related class 1 KNOX homeobox genes, in 6b-expressing transgenic *Arabidopsis* and *Nicotiana* plants. Protein 6b is found in nuclei and binds to several plant nuclear proteins. The role of 6b remains to be determined. Recently, we have found that 6b binds specifically to histone H3 in vitro but not to other core histones. Analysis by bimolecular fluorescence complementation revealed an interaction in vivo between 6b and histone H3. We recovered 6b from a chromatin fraction from 6b-expressing plant cells. A supercoiling assay and digestion with micrococcal nuclease indicated that 6b acts as a histone chaperone with the ability to mediate formation of nucleosomes *in vitro*. Mutant 6b, lacking the carboxy-terminal region that is required for cell division-stimulating activity and interaction with histone H3, was deficient in histone-chaperone activity. Our results suggest a relationship between alterations in nucleosome structure and the expression of growth-regulating genes on the one hand and the induction of aberrant cell proliferation on the other. Ref: Terakura et al., PLANT CELL 19: 2855-65 (2007)

Title: A small heat-shock protein HspL is induced by *virB* and involved in tumorigenesis in *Agrobacterium tumefaciens*

Author(s): Yun-Long Tsai, Ming-Hsuan Wang, and Erh-Min Lai

Abstract: We characterize a small heat-shock protein HspL, which was induced by a potent virulence (*vir*) gene inducer acetosyringone (AS) depending on the VirA/VirG two-component system in *Agrobacterium tumefaciens*. Regulation analyses indicated that AS-induced HspL protein accumulation is first upregulated at transcriptional level and then controlled at translational and/or posttranslational level to increase its protein accumulation in the presence of AS. Further expression analyses in various *vir* mutants suggest that AS-induced *hspL* transcription is not directly activated by VirG response regulator, rather dependent on the expression of *virB* genes, encoding the components of type IV secretion system (T4SS). Among the 11 *virB* genes encoded by the *virB* operon, HspL protein level is reduced in strains with deletion of *virB6*, *virB8*, and *virB11* respectively but not significantly affected by the rest of *virB* deletion mutants. Overexpression of VirB6 or VirB11 but not VirB8 in the absence of tumor-inducing (Ti) plasmid induces HspL expression to the level of Ti-plasmid containing strain induced by AS. All analyzed VirB proteins accumulated at lower levels in *hspL* deletion mutant at early stage of AS induction with most dramatic effects on VirB1, VirB2, VirB9, and VirB11. The deletion of *hspL* also results in 20-25% reduction of tumorigenesis efficiency on potato tuber discs and the deficiency in conjugal transfer of an IncQ plasmid RSF1010 derivative between *A. tumefaciens* strains. Both molecular and phenotypic changes caused by the deletion of *hspL* were restored to or greater the level of wild type level in complemented strain. Taken together, AS-induced HspL protein accumulation is induced at both transcriptional and translational/posttranslational in a *virB*-dependent manner. We suggest that the *virB*-induced HspL protein is involved in VirB protein expression/stability; leading to efficient DNA transfer and tumorigenesis.

Title: ***Agrobacterium rhizogenes* GALLS gene encodes two secreted proteins required for gene transfer to plants**

Author(s): Larry D. Hodges, Lan-Ying Lee, Stanton B. Gelvin, and Walt Ream

Abstract: *Agrobacterium tumefaciens* and *A. rhizogenes* are related pathogens that cause crown gall and hairy root diseases, which result from integration and expression of bacterial genes in the plant genome. Single-stranded DNA (T-strands) and virulence proteins are translocated into plant cells by a type IV secretion system. VirD2 nicks a specific DNA sequence, attaches to the 5' end, and pilots the DNA into plant cells. *A. tumefaciens* translocates single-stranded DNA-binding protein VirE2 into plant cells where it binds T-strands and targets them into the nucleus. Although some strains *of A. rhizogenes* lack VirE2, they transfer T-strands efficiently due to the GALLS gene, which complements an *A. tumefaciens* virE2 mutant for tumor formation. Unlike VirE2, full-length GALLS (GALLS-FL) contains ATP-binding and helicase motifs similar to those in TraA, a strand transferase involved in conjugation. GALLS-FL and VirE2 contain nuclear localization sequences and C-terminal secretion signals. Mutations in any of these domains abolish the ability of GALLS to substitute for virE2. GALLS encodes two proteins from one open reading frame: full-length and a C-terminal domain (GALLS-CT), which initiates at an internal in-frame start codon. On some hosts, both GALLS proteins were required to substitute for VirE2. GALLS-FL tagged with yellow fluorescent protein (YFP) localized to the nucleus of tobacco cells, whereas GALL-CT::YFP remained in the cytoplasm. In plant cells, the GALLS proteins interacted with themselves, VirD2, and each other. VirD2 interacted with GALLS-FL inside the nucleus where its predicted helicase activity may pull T-strands into the nucleus.

Agrobacterium Genomics

Title: Using Bioinformatics to Reconstruct the Origin of Multichromosome Bacterial Genomes

Author(s): Brad Goodner & the *Agrobacterium* Genome Consortium*

Abstract: The historic model of a bacterial genome was dominated by the *E. coli* "viewpoint" - a single circular chromosome with the possibility of one to a few plasmids. In the past two decades and especially since the advent of genomic sequencing, we have come to realize that chromosomes differ widely in size (<0.5 Mbp to > 10 Mbp) but also that they can differ in topology (linear chromosomes in *Streptomyces, Borrelia*, and some *Agrobacterium*) and in number. Two or three chromosomes can be found in members of several diverse genera across the Bacteria domain. While the additional chromosomes of these diverse genera did not all arise in one common event and do not all share broad sequence homology, they all do appear to share a common mechanism. There are no known examples of a chromosome in the Bacteria domain having more than one origin of replication (a few examples now known i n the Archaea domain), so simple breakage-and-recircularization of a single chromosome into two has not occurred. Rather, plasmids have acted as nucleation centers for the formation of additional chromosomes through intragenome gene transfers. The availability of hundreds of complete genome sequences allows for the detection and evolutionary reconstruction of such events. I will present evidence for 3 examples, including the Rhizobiales, based on comparisons of whole genomes.

*The Agrobacterium Genome Consortium is responsible for sequencing, assembly, and annotation of the *A. vitis* S4, *A. radiobacter* K84, and *A. rhizogenes* A4 genomes, and for the reannotation of the *A. tumefaciens* C58 genome ("gold standard"). The consortium consists of the following PI's and their lab groups: Gene Nester (University of Washington) Derek Wood (Seattle Pacific University), Barry Goldman (Monsanto Co.), Stephen Farrand (University of Illinois), Steve Slater (Arizona State University), Joao Setubal (Virginia Tech University), Tom Burr (Cornell University), Brad Goodner (Hiram College), Lois Banta (Williams College), Allan Dickerman (Virginia Tech University) & Leon Otten (University Louis Pasteur Strasbourg).

Title: The evolution of the *repABC* genes from three sequenced *Agrobacterium* species is incongruous with their cognate plasmid and each other.

Author(s): Barry Goldman & the *Agrobacterium* Genome Consortium*

Abstract: Many of the Alpha-proteobacteria have replicons that require the RepABC proteins for replication. It is well know that plasmids are often derived from horizontal gene transfer (HGT). To understand the evolution of the plasmids found in *Agrobacterium vitis, A. radiobacter* and *A. tumefaciens* we have performed a phylogenetic analysis of their associated RepABC proteins. To do this, the RepA, B, and C proteins of the *A. tumefaciens, A. radiobacter* and *A. vitis* from all plasmids were aligned using the Muscle algorithm. Bootstraps and trees were generated using the Mega 3.1 algorithm. Analysis of the RepC proteins suggests that their evolution is incongruous with the evolution of the plasmids. For example, the pC2 plasmid of *A. vitis* and the linear plasmid of *A. tumefaciens* are considered chromosomes, as both have ribosomal DNA regions and essential proteins. Yet, the RepC proteins of each plasmid do not derive from a recent common ancestor. In addition, the evolution of the RepA and RepB proteins from each plasmid are incongruous with the evolution of the RepC protein. The data show however, that the RepA and RepB proteins are evolutionarily congruent. The genes can not be used for phylogenetic analysis, suggesting a distant evolutionary history. We also note that the *A. vitis* genome, which contains 7 replicons, has two extra *repA* and *repB* genes (on the p79 and p259 plasmids) and the *A. radiobacter* genome, which contains 5 replicons, has an extra copy of these genes on the p388 plasmid.

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Title: Genome sequencing of *Agrobacterium* biovars: the genome of *Agrobacterium rhizogenes* A4

Author(s): Steve Slater, Kathryn Houmiel, Erika Frederick &the *Agrobacterium* Genome Consortium*

Abstract: Since 2001 the genomes of *Agrobacterium tumefaciens C58, A. radiobacter* K84 and *A. vitis* S4 strains have been sequenced. These organisms represent all three biovars and showcase the diversity of the genus. In order to investigate variations between biovar II species that pursue pathogenic and biological control lifestyles we have initiated a project to sequence the genome of the hairy root pathogen *Agrobacterium rhizogenes* A4. The A4 genome is comprised of two large circular replicons and two plasmids with an estimated total size of ~7.3MB. The genome has been sequenced to 40x coverage using 454 pyrosequencing technologies. Finishing is being done using a combination of optical mapping, comparison with the related genome of K84 and the construction and sequencing of 384 paired fosmid reads. Initial findings have identified the presence of a widely conserved type III secretion system found in most biovar II strains. In addition, comparisons with the genome of K84 suggest that while the main chromosome of A4 and K84 are highly syntenic, there is only limited similarity between the second replicon of A4 and the 2.65Mb replicon of K84. These findings are consistent with the proposed evolution of large second replicons from plasmids among the Rhizobiaceae. This project is operated as part of a larger undergraduate training program in genomics at Hiram College, Arizona State University and Seattle Pacific University. This approach to genome sequencing has proven to be an efficient and cost effective way to finish microbial genomes.

*The Agrobacterium Genome Consortium is responsible for sequencing, assembly, and annotation of the *A. vitis* S4, *A. radiobacter* K84, and *A. rhizogenes* A4 genomes, and for the reannotation of the *A. tumefaciens* C58 genome ("gold standard"). The consortium consists of the following PI's and their lab groups: Gene Nester (University of Washington) Derek Wood (Seattle Pacific University), Barry Goldman (Monsanto Co.), Stephen Farrand (University of Illinois), Steve Slater (Arizona State University), Joao Setubal (Virginia Tech University), Tom Burr (Cornell University), Brad Goodner (Hiram College), Lois Banta (Williams College), Allan Dickerman (Virginia Tech University) & Leon Otten (University Louis Pasteur Strasbourg).

Poster Abstracts:

Title: Analysis of Tetracycline Resistance and Interruption of the Counterselection Marker *sacB* by IS426, an *Agrobacterium tumefaciens* C58 Insertion Sequence

Author(s): Cheríe Blair, Peter M. Merritt, and Clay Fuqua

Abstract: The *Agrobacterium tumefaciens* C58 genome contains three copies of IS426, a 1.3 kb insertion sequence. Previous studies revealed that insertion of IS426 into the *tetR* locus derepresses expression of the endogenous *tetA* tetracycline resistance (*TcR*) gene in *A. tumefaciens* C58, resulting in spontaneous TcR. In various bacteria, allelic replacement mutagenesis utilizes the conditionally-lethal levansucrase gene *sacB* to select against plasmid insertion mutants that retain the integrated plasmid. Levansucrase produces fructosyl levans in the periplasm when gram-negative cells are grown on sucrose. The lethality of levan accumulation is used to identify integrants in which a second recombination event has excised the plasmid, enabling sucrose resistance (SucR). In C58, use of *sacB* counterselection is complicated by a high frequency, approximately 1 per 10^6 integrants, of spontaneous SucR mutants that have not excised the plasmid. This suggested a high loss of function mutation rate in the *sacB* gene. To determine the nature of these putative mutations, we PCR amplified the *sacB* gene in SucR mutants that retained the plasmid marker. Many mutants yielded products approximately 1.3 kb longer than wild type *sacB*, and sequencing revealed IS426 insertions. Southern blot analysis of several SucR-*sacB* and spontaneous TcR mutants revealed the three expected copies in the C58 genome and a fourth copy in *sacB* and *tetR*, respectively. The exact site of insertion in *sacB* appears to vary. IS426 is clearly highly active in C58 and can often complicate otherwise effective selection strategies.

Title: Blocking T-strand conversion to double-stranded intermediates by expression of single-stranded DNA binding proteins

Author(s): Mery Dafny-Yelin, Raz Dafny, Lorenzo Prieto, Avner Levy and Tzvi Tzfira

Abstract: *Agrobacterium* delivers its T-strand into the host-cell nucleus where it can be converted into double-stranded molecules. Various studies have revealed that double-stranded (ds) T-DNA intermediates can serve as substrates by an as yet uncharacterized integration machinery. Nevertheless, the possibility that T-strands can still be the substrates for integration cannot be excluded. To further investigate the route taken by T-DNA molecules on their way to integration, we attempted to block the conversion of T-strands to double-stranded intermediates prior to integration. We produced transgenic plants which overexpressed three protein subunits of DNA Replication Factor-A (RFA) from yeast. These subunits, RFA1, RFA2 and RFA3, function as a complex in yeast cells which is capable of binding to single-stranded (ss) DNA molecules, promoting the repair of double-strand breaks. RFA overexpression in *Nicotiana bentamiana*, however, resulted in increased sensitivity to DNA-damaging agents relative to wild-type plants, indicating that heterologous and high expression of RFA may interfere with the host DNA-repair machinery, most likely by interacting with exposed DNA strands at genomic break sites. RFA-transgenic plants were defective in T-DNA expression, as determined by infection with *Agrobacterium* cells carrying the GUS intron reporter gene. Gene expression was not blocked when the reporter gene was delivered into plant cells by microbombardment. Confocal microscopy analysis revealed that yeast RFA forms a complex which resides within the plant nuclei and preliminary data showed that the cell-to-cell movement of the ssDNA bean dwarf mosaic virus is defective in RFA plants. These observations suggest that RFA may interfere with the T-strand's conversion to its double-stranded form, and by implication, block its expression and integration into the host genome.

Title: Peptide aptamers for defining protein function

Author(s): Stanton B. Gelvin, Zhuzhu Zhang, and Lan-Ying Lee

Abstract: Peptide aptamers (from the Latin aptus for "fitting") are short peptides of random sequence that can interact with specific target proteins in vivo. As commonly used, these peptides are generally 15-20 amino acids-long. This length provides enough flexibility for the peptide to assume various conformations while reducing the probability of randomly creating a stop codon in the aptamer coding sequence. We are currently developing peptide aptamer "mutagenesis" technology for use in plants. As proof of concept, we are targeting VirE2 with various 20-mers from the VirE2 protein sequence. VirE2 is known to interact with VirE1, VirE2, importin α, and VIP1; for many of these protein pairs, sites important for interaction with VirE2 are known. We have designed an aptamer expression cassette, based upon pSAT vectors, which uses bimolecular fluorescence complementation to detect aptamer-protein interactions. The cassette consists of a CaMV double 35S promoter + full-length mCherry + a multiple cloning site for insertion of aptamer coding sequences + nVenus + CaMV polyA addition signal. We have individually tagged VirE2 with cCFP and nCerulean. Dimerization of VirE2 brings together cCFP and nCerulean, generating blue fluorescence. Interaction of VirE2-nCerulean with the mCherry-aptamer-nVenus polyprotein generates yellow fluorescence. Expression of the aptamer cassette results in red mCherry fluorescence. Interaction of the aptamer with VirE2 may inhibit VirE2 function, resulting in decreased transformation of plant cells expressing the aptamer. We are testing these constructions in tobacco BY-2 cells and transgenic *Arabidopsis*. Preliminary results indicate that multiple aptamer polyproteins can interact with VirE2 in tobacco cells.

Title: A Functional Genomics Clearinghouse as an Outcome of Undergraduate & High School Education – An Update of Efforts at Hiram College

Author(s): Brad Goodner[1], Cathy Wheeler[1], Prudy Hall[1], Stuart Gordon[1], Kathryn Reynolds[1], Stephanie Lammlein[2], 2002-2007 Molecular & Cellular Biology courses[1], 2002-2007 Genetics courses[1], 2006-2007 Introductory Biology courses[2], & 2006-2007 Hiram Genomics Academy sessions[3]

Abstract: Obtaining the complete genome sequence of any organism is really just a new beginning – you have a lot of tools now available but are not yet sure how best to use them. Functional genomics is really just an extension of how molecular biology and microbiology have made progress over the past 25 years or more through characterization of mutants through forward or reverse genetics. The extension is one of breadth to include as many genes as possible. Over the past 6 years, Hiram College faculty and students have accumulated a large set of mutations in the *A. tumefaciens* C58 genome (and to a lesser extent in the *A. rhizogenes* A4 genome). Students in the Molecular & Cellular Biology course use reverse genetics to test functional predictions based on bioinformatics analyses of gene function. We will highlight the work done by the 2006 and 2007 iterations of the course. Students in the Genetics course, along with high school students during the past two years, have used forward genetics to link genes to functions through a large scale mutant hunt. We will highlight some of the more interesting findings in several functional categories. Finally, we will look forward to future efforts and we are certainly open to new suggestions from others.

Title: Finishing the Genome of *Agrobacterium rhizogenes* A4 Using a Combination of 454 Sequencing and Optical Mapping

Author(s): Erika Frederick[1], Kathryn Houmiel[2], Derek Wood[2] & the *Agrobacterium* Genome Consortium*

[1]Stanford University, Stanford, CA 94305 [2]Department of Biology, Seattle Pacific University, Seattle, WA 98053

Abstract: *Agrobacterium rhizogenes* A4, an agropine type pathogen, induces a hairy root phenotype on host plants. Its genome contains two circular chromosomes and 2 plasmids. The genome recently has been sequenced and is being finished using a combination of optical mapping, fosmid end sequencing, synteny with *A. radiobacter* K84, and traditional gap filling technology. Sequencing and assembly using 454 Sequencing yielded a total of 124 contigs. An optical map of A4, generated by Opgen, permitted multiple gaps to be closed and decreased the number of contigs to 72. Comparison of the gene arrangement of *A. radiobacter* K84 to that *A. rhizogenes* A4 further decreased the number of contigs to 64. Fosmid clones created by gDNA shearing, size selection for DNAs 2-25 kb, and end sequencing gave 384 paired end reads, provided additional scaffolding information. Sequencing contig overlaps, only 15 bp in length, permitted confirmation of sequence identity at these points. Use of fosmid clones and gap filling technologies, reduced the number of contigs reduced to 32. Standard sequencing methods will be used to fill the remaining gaps so that the genome will be completely closed. The assembly work was accomplished by an undergraduate student over the course of last summer and demonstrates a more efficient and cost effective way to finish microbial genomes, as opposed to using standard sequencing technologies alone.

*The Agrobacterium Genome Consortium is responsible for sequencing, assembly, and annotation of the *A. vitis* S4, *A. radiobacter* K84, and *A. rhizogenes* A4 genomes, and for the reannotation of the *A. tumefaciens* C58 genome ("gold standard"). The consortium consists of the following PI's and their lab groups: Gene Nester (University of Washington) Derek Wood (Seattle Pacific University), Barry Goldman (Monsanto Co.), Stephen Farrand (University of Illinois), Steve Slater (Arizona State University), Joao Setubal (Virginia Tech University), Tom Burr (Cornell University), Brad Goodner (Hiram College), Lois Banta (Williams College), Allan Dickerman (Virginia Tech University) & Leon Otten (University Louis Pasteur Strasbourg).

Title: Quantitative measurements of cell attachment of *Agrobacterium tumefaciens* to *Arabidopsis thaliana* with the use of a Flow Cytometer.

Author(s): Anna Petrovicheva, Leslie Aguirre, Khudeja Mir, Nel Trasybule, Lourdianie P-Charles and Theodore R. Muth

Abstract: *Agrobacterium tumefaciens* is a bacterium that is able to infect a diverse array of plants. It attaches to plant roots and transforms the plants cell to induce crown gall tumors, a type of plant cancer. The plants used in this experiment are *Arabidopsis thaliana*, a member of the mustard family. The goal is to create an efficient quantitative measurement assay that improves upon the past methods. Agro infection assays using transformation leading to fluorescence and tumor formation are informative, but these do not measure actual attachment. The previous attachment assays that have been done using microscopy, give a more qualitative than quantitative result, and colony counts are labor intensive and measure only the numbers of living bacteria that can be released from roots. Some of the attached bacteria may be killed, or enter a dormant state, in the preparation process. The flow cytometer measures the amount of particles in a media sample and determines their size and fluorescence in a data sheet of the program. Using the flow cytometer, the number of bacteria extracted from the root surface will be measured. From this data, we can identify mutant bacterial clones, or mutant *Arabidopsis* lines, that exhibit enhanced attachment or inhibited attachment.

Title: A Novel Type VI Secretion System is Linked to Biofilm Formation in *Agrobacterium tumefaciens*

Author(s): David Rogawski[1], Amelia Tomlinson[2], Clay Fuqua[2], and Lois Banta[1]

[1]Department of Biology, Williams College, Williamstown, MA 01267
[2]Department of Biology, Indiana University, Bloomington, IN 47405

Abstract: The fourteen genes of the *imp* operon in *Agrobacterium tumefaciens* encode a putative Type VI secretion system (T6SS). T6SS gene clusters are highly conserved and are found in one or more copies in a variety of pathogenic Gram-negative bacterial species. Type 6 Secretion was first classified in *Vibrio cholerae*, where it was shown to be a major virulence determinant and to transport proteins lacking N-terminal hydrophobic signal sequences (Pukatzki, et al., Proc. Natl. Acad. Sci. USA 103:1528; 2006). Genes that encode proteins with homology to serine-threonine kinases and phosphatases are components of many T6SS loci, and in *Pseudomonas aeruginosa* the kinase is required for secretion while the phosphatase downregulates secretion (Mougous, et al., Science 312:1526; 2007). To determine the function of the T6SS in *A. tumefaciens*, we obtained mutants in several of the *imp* genes, including the putative kinase and phosphatase regulator genes, as well as a putative substrate *vrgG*. Compared to the wild-type strain C58, biofilm formation was increased in an *imp* operon deletion mutant over forty-fold, as determined by crystal violet staining of biofilms on PVC plates. Flow cell analysis and confocal microscopy reveal more prevalent, and substantially larger microcolonies for the *imp* mutant as compared to the wild type. Interestingly, biofilm formation is increased by a comparable amount in both the kinase and phosphatase regulator mutants, but not in the *vrgG* mutant. We propose that the T6SS secretes an inhibitor of biofilm formation. Our data further suggest that the kinase and phosphatase may regulate the secretion of an additional inhibitor that acts upon the T6SS system itself; in the absence of kinase or phosphatase function, the inhibitor might prevent the T6SS from functioning, resulting in the same biofilm phenotype as the imp operon mutant. Quorum sensing has been shown to regulate biofilm formation in several bacterial species. We hypothesized that the T6SS in *A. tumefaciens* may regulate or respond to a quorum sensing system that is responsible for its effect on biofilm formation. To test this hypothesis we added conditioned media from imp mutant and wild-type strains to mutant and wild-type cultures growing in PVC wells; the results of these experiments will be presented. Expression of the *imp* operon is increased in the presence of conditioned media from wild-type cells, further suggesting that the T6SS may be implicated in quorum sensing.

Title: ***Agrobacterium*** **attachment to** ***Arabidopsis*** **roots induces the systemic expression of a defense-response gene, PAL1**

Author(s): Nagesh Sardesai, Veena, Clay Fuqua, and Stanton B. Gelvin

Department of Biological Sciences, Purdue University, West Lafayette, IN 47907
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Abstract: *Agrobacterium*-mediated transformation is a result of a complex interaction between the bacterium and the host plant. However, little information is available about the factors responsible for biofilm formation on the plant root surface, and defense responses mounted by the plant against the perceived pathogen attack. The formation of biofilms and host defense responses may be linked. The *Arabidopsis* defense-response gene phenylalanine ammonia lyase (PAL1) is up-regulated in response to infection by *Agrobacterium*. We fused the PAL1 promoter with EYFP and generated transgenic *Arabidopsis* containing this transgene. PPAL-EYFP plants expressed the PAL1 promoter in the root vasculature. We used mCherry-tagged wild-type and mutant *A. tumefaciens* strains to investigate interactions between the bacteria and the plant. *A. tumefaciens* C58 could form biofilms on *Arabidopsis* roots by 6 hours after inoculation. When PPAL-EYFP plants were infected with high concentrations of *A. tumefaciens*, the PAL1 promoter expressed in root epidermal cells at different times depending on which *Agrobacterium* strain was used. *An A. tumefaciens* strain over-expressing the SinR transcription factor induced PPAL-EYFP expression in root epidermal cells at colonization sites as early as two hours. A *motA A. tumefaciens* mutant induced a systemic response in leaves 24 hours after infection. This was the only tested strain that induced a systemic response. We are currently investigating the extent and kinetics of PAL1 promoter response to *Agrobacterium* in various *Arabidopsis* mutants altered in their defense responses.

Title: Characterization of *Arabidopsis* mutants that are hyper-susceptible to *Agrobacterium*-mediated transformation (*hat* mutants)

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Abstract: We used activation tagging to identify *Arabidopsis* mutants that are hyper-susceptible to *Agrobacterium* transformation. We identified eight such hat mutants, and isolated T-DNA/plant junctions from five of them. The *hat1* mutant contains a T-DNA insertion in the 7th exon of a gene encoding a cellulose synthase-like (CSL) protein. RT-PCR indicated that CSL was down-regulated whereas a neighboring gene, UDP-glucosyl transferase (UGT), was strongly up-regulated. Over-expressing a UGT cDNA in wild-type plants resulted in increased root transformation. Knockdown mutants of CSL also showed a mild hat phenotype. The hat3 mutant has a T-DNA insertion in the 5' untranslated region of a *myb* transcription factor (MTF). RT-PCR analyses revealed low MTF transcript levels. Three MTF T-DNA insertion mutants showed increased transformation but MTF over-expressing plants did not show decreased transformation. We are currently conducting microarray analysis of wild-type, MTF over-expressing, and MTF knockdown plants to identify genes regulated by MTF. Two independent mutants, hat4 and hat7, have T-DNA insertions in the 5' region of a potassium transporter family (PTF) gene but did not show alterations in PTF transcripts. None of the genes in the proximity of this T-DNA showed differential regulation. The hat5 mutant has a T-DNA insertion in the intergenic region between a thioredoxin gene and an integral membrane transporter family (IMTF) gene, with high levels of transcript accumulation for both genes. Thioredoxin over-expressing plants did not recapitulate the hat phenotype. We are currently generating IMTF over-expressing transgenic plants to assess the importance of this gene in transformation.

Title: Expression of the Arabinogalactan-protein gene, AtAGP17, in recalcitrant *Arabidopsis* Bl-1 ecotype results in increased sensitivity to *Agrobacterium*-mediated transformation

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Abstract Arabinogalactan-proteins (AGP) are a family of complex highly glycosylated hydroxyproline-rich glycoproteins that are expressed throughout the plant kingdom. Mutation of the arabinogalactan-protein gene AtAGP17 results in decreased *Agrobacterium*-mediated root transformation of Arabidopsis ecotype Ws-2. Roots of the AtAGP17 mutant bind *Agrobacterium* poorly. We introduced the AtAGP17 gene into the Arabidopsis ecotype Bl-1, which is highly recalcitrant to root transformation by *A. tumefaciens. Agrobacterium* cells bind poorly to roots of this ecotype. Transgenic lines showed increased efficiency of both transient and stable root transformation. RT-PCR analyses revealed varying levels of AGP17 transgene transcripts in these lines. Transgenic AGP17-expressing Bl-1 lines showed increased attachment of wild-type *A. tumefaciens* C58, as visualized by binding assays using fluorescently-tagged bacteria. Our studies reveal a possible important role for AGP17 in root transformation of *Arabidopsis*.

Title: The importance of core histone genes for *Agrobacterium*-mediated plant transformation

Author(s): Joerg Spantzel, Gabriela N. Tenea, Yanmin Zhu, Simran Bhullar, and Stanton B. Gelvin

Abstract: We previously showed that introduction of additional copies of the Arabidopsis histone H2A-1 gene HTA1 or its cDNA increases the transformation frequency of *Arabidopsis* and several other plant species. This was demonstrated both in stably transformed transgenic plants and with the HTA1 gene transiently expressed from an incoming T-DNA. We also reported that infection of tobacco BY-2 cells with a transformation-proficient *Agrobacterium* strain induces the expression of all families of core histone genes (histone H2A [HTA], histone H2B [HTB], histone H3 [HTR] and histone H4 [HFO]). The *Arabidopsis* genome contains 46 core histone genes which encode 33 different core histone proteins. We tested 20 representative core histone genes for their ability to enhance transformation. We expressed cDNAs of these genes under the control of a CaMV double 35S promoter in transgenic *Arabidopsis*. We then re-transformed roots of these transgenic plants with *Agrobacterium*, and compared the transformation frequency with that of wild-type control plants. Additional copies of all tested HTA and HFO genes and one HTR gene, but no HTB or most HTR genes, increased the frequency of *Agrobacterium*-mediated plant transformation. We obtained homozygous *Arabidopsis* plants with T-DNA insertions into the histone H2A genes HTA2, -3, -6, -8, -9, -10 and transformed them using *Agrobacterium*. Compared to wild-type control plants, we could not observe any difference in transformation frequency. These results suggest either that these genes do not play a role in transformation, or that they are functionally redundant with regard to transformation.

Title: Over-expression of *Arabidopsis* chromatin genes results in increased transformation efficiency and/or transgene expression

Author(s): Gabriela N. Tenea, Joerg Spantzel, Lan-Ying Lee, Susan Jonhson, Yanmin Zhou, Heiko Oltmanns and Stanton B. Gelvin

Abstract: Previous work from our laboratory indicated an essential role for Arabidopsis histone genes in T-DNA integration. RNAi targeted against 109 Arabidopsis chromatin genes further demonstrated a role for other chromatin proteins, such as SGA1, in *Agrobacterium*-mediated transformation. We investigated the effects of over-expressing numerous *Arabidopsis* histone cDNAs and an anti-silencing factor A (SGA1) cDNA on transformation and transgene expression. Transgenic *Arabidopsis* plants containing additional copies of cDNAs encoding histone H2A (HTA), histone H4 (HFO), or SGA1 displayed increased susceptibility to transformation. Over-expression of all tested histone H2B (HTB) and most histone H3 (HTR) cDNAs did not increase transformation. A parallel increase in transient gene expression was observed when the histone HTA or HFO cDNAs were co-transfected, together with a plant active *gusA* gene, into tobacco protoplasts. Using RT-PCR, we also detected an increase in *gusA* transcripts when the histone HTA1 cDNA was over-expressed in protoplasts. No such increase in gusA activity was seen when a SGA1 cDNA was co-transfected with a *gusA* gene into BY-2 protoplasts. These results suggest that over-expression of HTA and HFO increases transformation by stimulating transgene expression, whereas over-expression of SGA1 increases transformation by a different mechanism. Over-expression of histone or SGA1 cDNAs does not increase expression of a previously integrated transgene, nor could HTA1 reverse silencing. These data suggest that histones may increase transgene expression by working directly on the promoter of incoming DNA, or that histones may play a role in stabilizing transgene DNA (and thereby transgene expression) during the initial stages of transformation.

Title: T-DNA trapping by genome-wide double-strand DNA breaks

Author(s): Amanda S. Freed and Tzvi Tzfira

Abstract: Although several different cellular pathways have been suggested to describe T-DNA's integration into plant cells, the mechanism by which T-DNA molecules integrate into the host genome is still largely unknown. Recent reports have revealed the roles played by DNA repair and maintenance proteins during the integration process. More specifically, the critical roles played by KU80, a nonhomologous end-joining host protein, and genomic double-strand breaks (DSBs) in T-DNA integration have led to the notion that T-DNA molecules can integrate as double-stranded intermediates. To deepen our understanding of the integration of T-DNA molecules into various genomic locations, a method for site-specific induction of DSBs was needed. We reasoned that transient expression of a restriction enzyme, capable of producing multiple genomic DSBs, would be useful for analyzing T-DNA integration into multiple genomic locations. To this end, we harnessed the power of AscI, an 8-base restriction enzyme capable of recognizing ca. 80 sites in the *Arabidopsis* genome. Proper engineering of this prokaryotic protein enabled its expression, translocation into the host cell nucleus and digestion of both extrachromosomal and genomic DNA. More importantly, coupling the expression of AscI with *Agrobacterium* infection resulted in the trapping of T-DNA molecules into AscI recognition sites in the *Arabidopsis* genome. Using a collection of primers which were designed to produce a unique amplification pattern for all genomic AscI recognition sites, we were able to monitor and detect the occurrence of DSB-site disruption and/or site-specific T-DNA integration events. We generated nearly a thousand independent T-DNA integration events and discovered that they were equally distributed throughout the *Arabidopsis* genome, regardless of AscI-site locations. We are currently plotting our DSB-mediated T-DNA integration distribution map against random T-DNA integration events, as mapped by the SALK T-DNA collection, and analyzing the statistical significance of our data in order to provide a model for DSB-mediated T-DNA integration.

Title: Crucial role of auxin and ethylene genes in *A. rhizogenes* induced hairy roots

Author(s): Veena, W. Kevin Lutke and Christopher G. Taylor

Abstract: *A. rhizogenes* is a Gram-negative, rod-shaped soil bacterium and is the causal agent for the development of the hairy root disease (also called root-mat disease) in plants. *A. rhizogenes* infection induces the formation of large numbers of transgenic roots with abundant lateral root branching. The Ri plasmid of K599 contains a single T-DNA with 11 open reading frames (ORFs). These open reading frames encode for three root-oncogenic loci (*rol*)-like genes (*rolA*, *rolB*, and *rolC*), a cucumopine synthase gene, plus other genes of unknown function. Previous models for *A. rhizogenes* root induction suggest that auxin production or changes in auxin sensitivities mediated by the *rol* genes may be the driving force in the production of hairy roots. Using microarrays we have analyzed the alterations in the global gene expression in *A. rhizogenes* induced hairy roots in *Arabidopsis* (composite plants). Comparisons between hairy and wild-type roots indicate that ethylene, auxin biosynthesis/transport genes may play an important part in the hairy root phenotype. Analysis of *Arabidopsis* mutants in auxin signaling and ethylene biosynthesis further confirms their involvement in the hairy root phenotype.

Title: Disarming *Agrobacterium rhizogenes*: A new tool for plant transformation

Author(s): Veena, Ray Collier, W. Kevin Lutke and Christopher G. Taylor

Abstract: Over the past three decades, the advancement in genetic engineering of plants with desired traits have relied heavily on the various strains of *A. tumefaciens*. However, many economically important plant species, and elite cultivars, are highly recalcitrant to *A. tumefaciens*-mediated transformation. *A. rhizogenes* strain K599 is highly infective in a broad range of plant species including legumes (soybean, alfalfa, Medicago) and transformation-recalcitrant crops including sweet potato and *Brassica*, thus making it a particularly useful strain for transformation purposes. With the use of homologous recombination approach, we have generated a disarmed strain of *A. rhizogenes* strain K599. This disarmed strain is missing the T-DNA encoded region and border sequences of the K599 Ri-plasmid and is completely free of any hairy root inducing properties. It is fully capable of transient and stable transformation of various plant species and posses several appealing characteristics, including higher transformation efficiencies, lower incidence of multiple T-DNA insertions and fewer vector backbone insertions. This disarmed stain of *A. rhizogenes* is an excellent alternative to *A. tumefaciens* for the genetic engineering of plants.

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