Rear and Release Psyllids as Biological Control Agents – An Economical and Feasible Mid-Term Solution for Huanglongbing (HLB) Disease of Citrus

2012 Specialty Crop Research Initiative Grant

$9,000,000 Award

CRDF Citrus Research and Development Foundation, Inc.

CITRUS RESEARCH BOARD

Requirements for Management

- ✔ Slow spread of disease – CHMA and insect control
- Treat existing infected trees
- Protect new plantings
- Provide long-term sustainable genetic and biological solutions

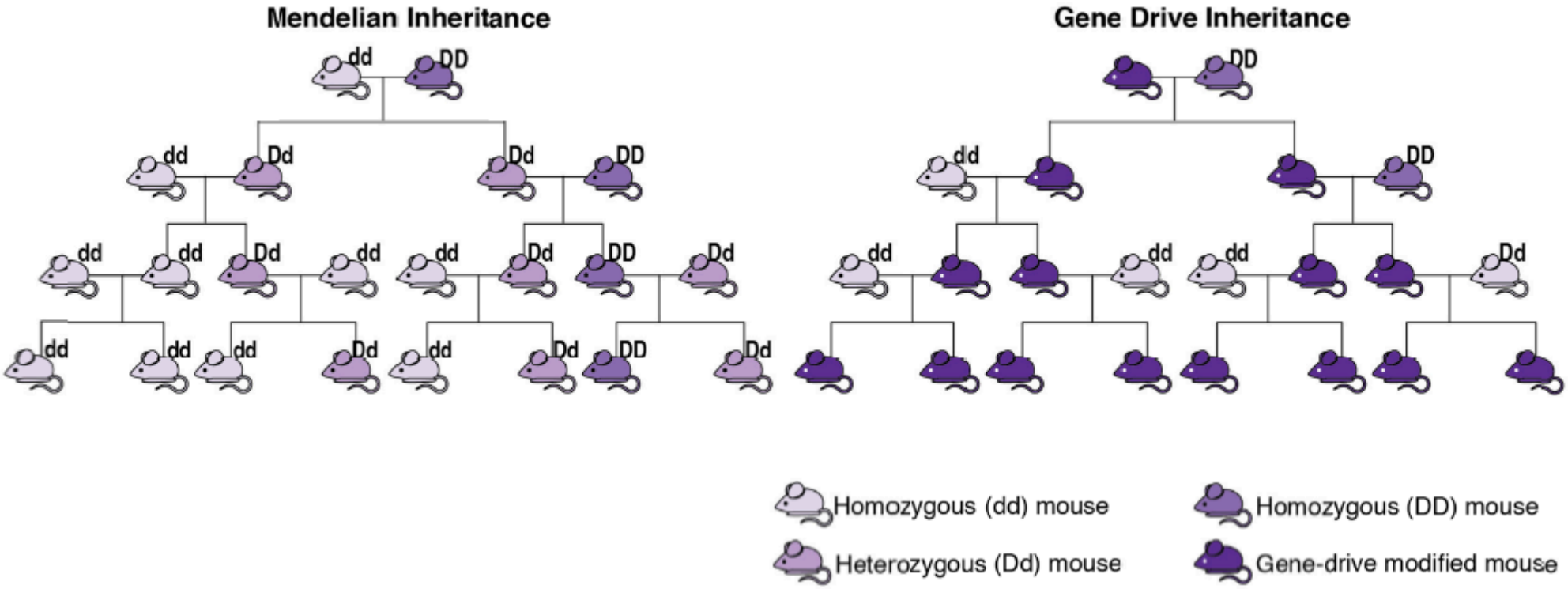
Advances in Insect Biotechnology for Pest Management

- Classical Biological Control
  - Parasitoids, predators, pathogens, competitors
- New Tools for Population Suppression
  - Sterile insect techniques using irradiation; example of screwworm, huge ROI from mitigation of risk ($200MM per year since ‘50s)
  - Synthetic biology improvements address both fitness reduction (somatic damage) and sex separation pioneered by Oxitec (now Intrexon)
- New Tools for Population Replacement
  - Gene drive
  - Synthetic biology improvements from gene editing

nuPsyllid

- Develop psyllid populations incapable of transmitting *C*Las (nuPsyllid) and strategically release the nuPsyllid population to replace current ACP populations that have invaded the US,
- Provide optimized management strategies for the integration of the proposed population replacement technique into current management practices,
- Integrate the management strategies with monitoring strategies to continually assess effectiveness, and
- Provide outreach education to the grower stakeholders and citizens about the control strategy.

Gene Drive – How do Genes Spread in Populations Without Fitness Selection?

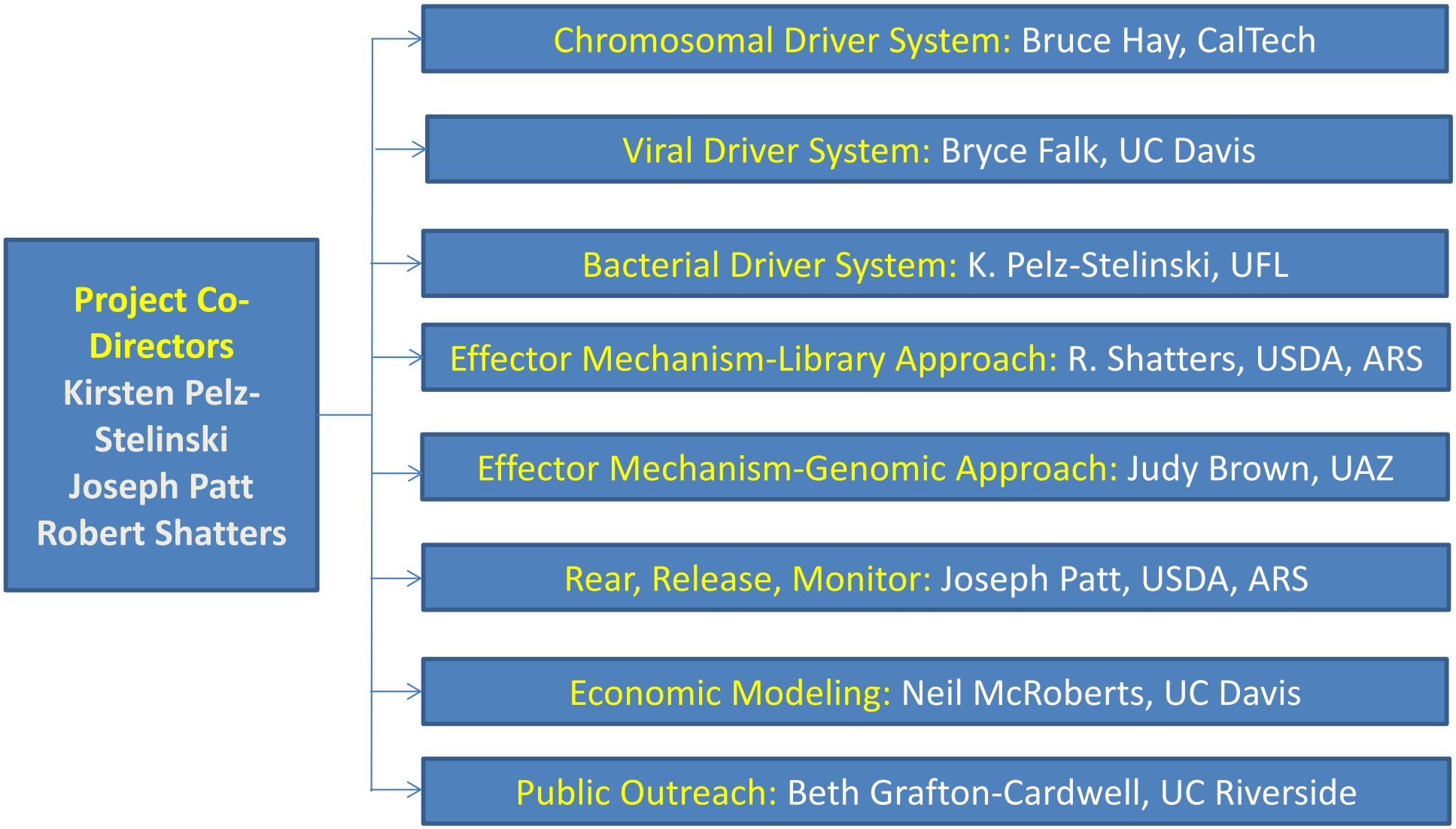


National Academies of Sciences, Engineering, and Medicine. 2016. Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values. Washington, DC: The National Academies Press.

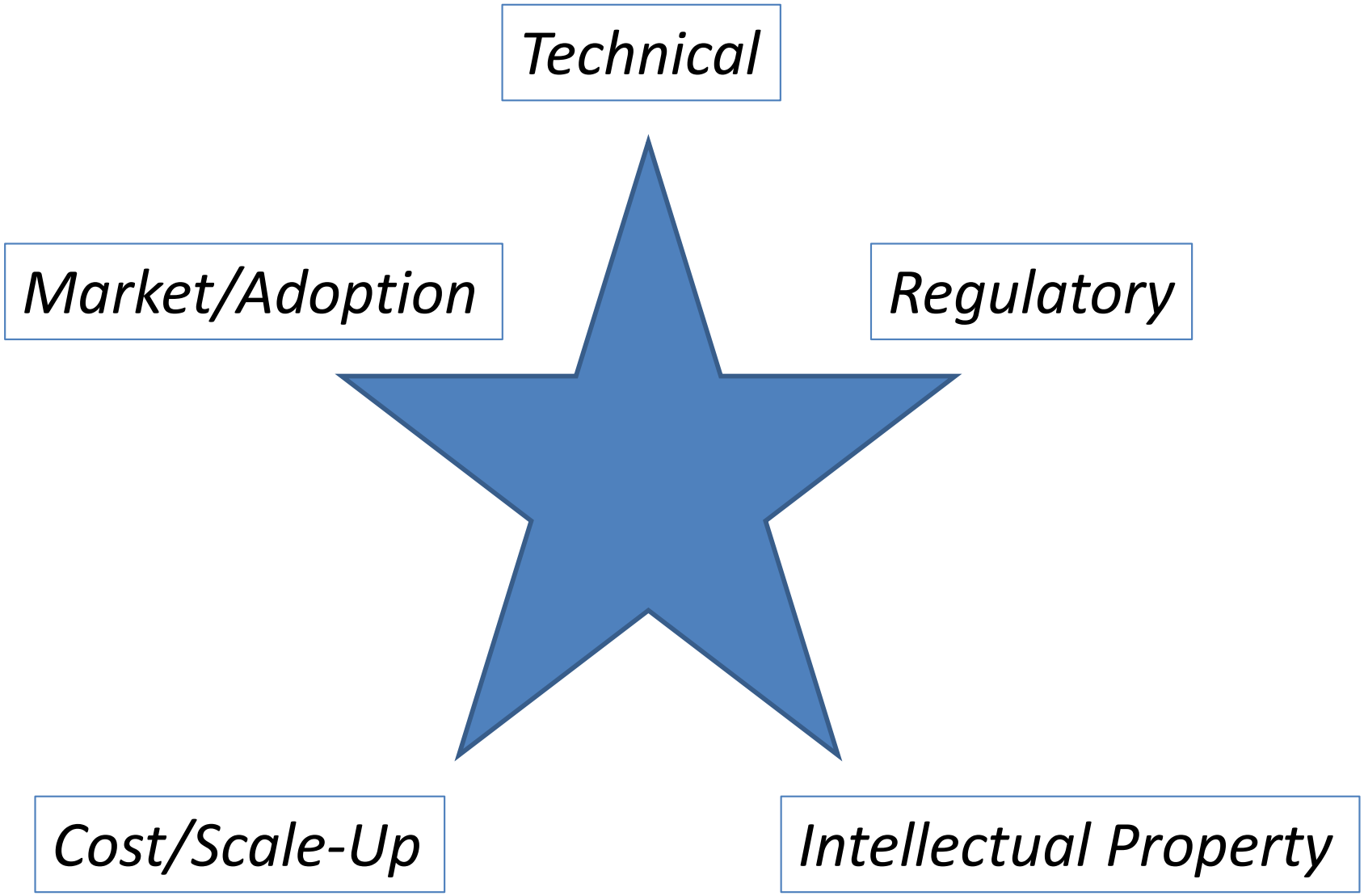
Project Team: Research & Delivery

- Composed of scientists from State Universities, State Departments of Agriculture, and USDA laboratories in many states, including Florida, Texas, California, Arizona, Oregon, Washington, and Wisconsin
- Includes biological, physical, and socio-economic expertise
- Coordinated to adapt current technological strategies showing success in other insect-vectored disease control campaigns

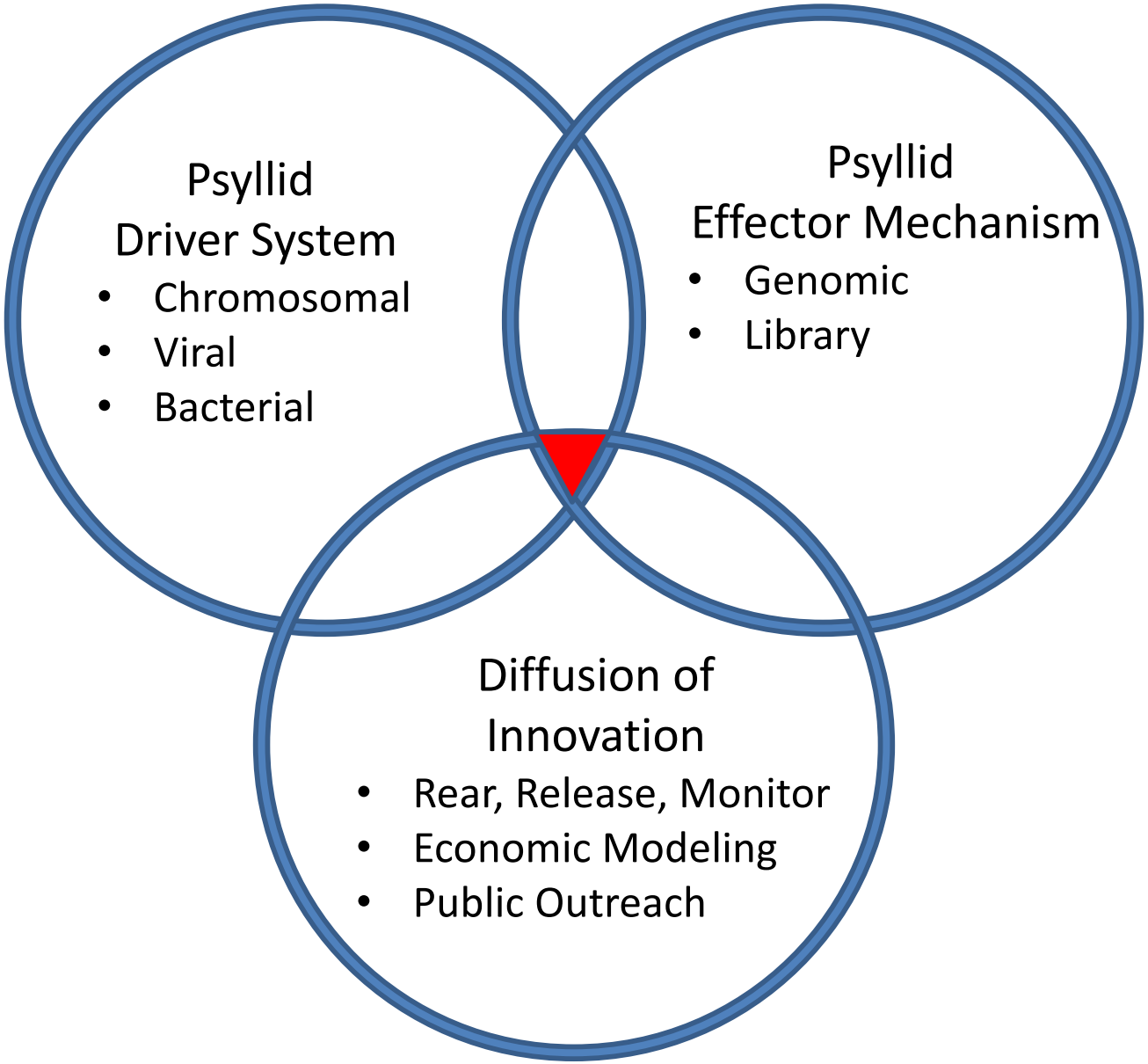
Research & Development Team



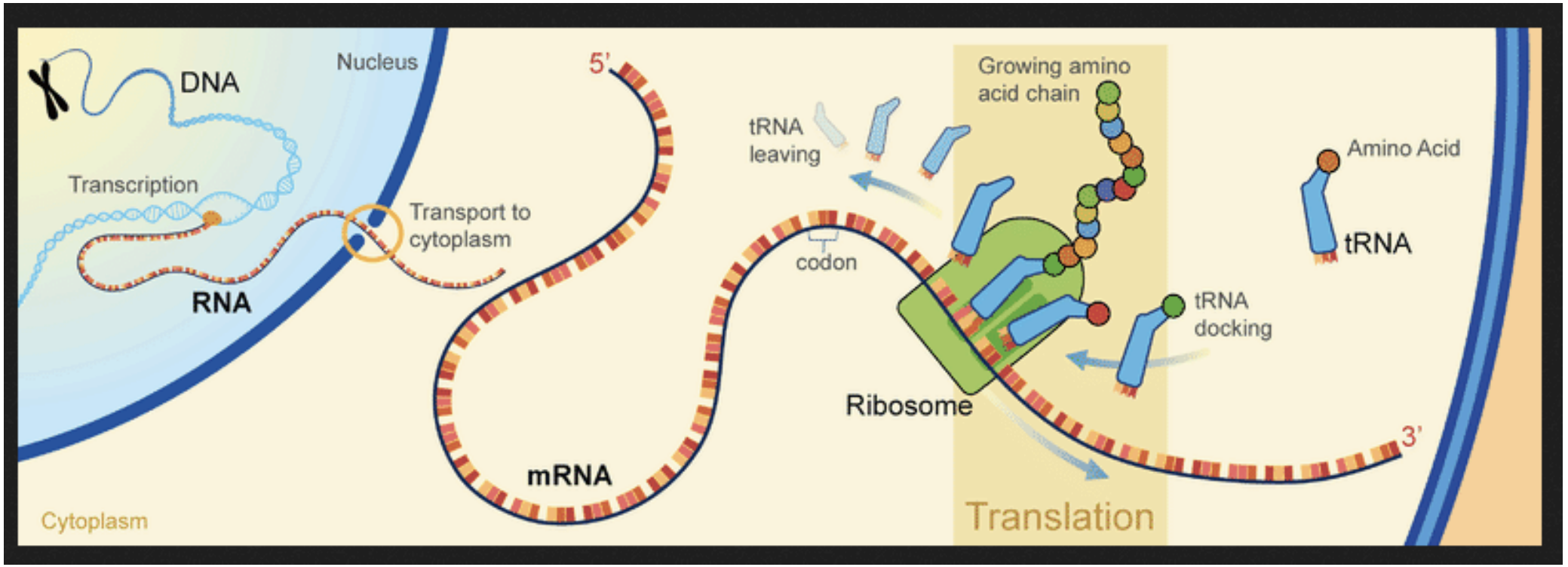
Commercial Delivery Happens When an Innovative Solution Has Been De-Risked



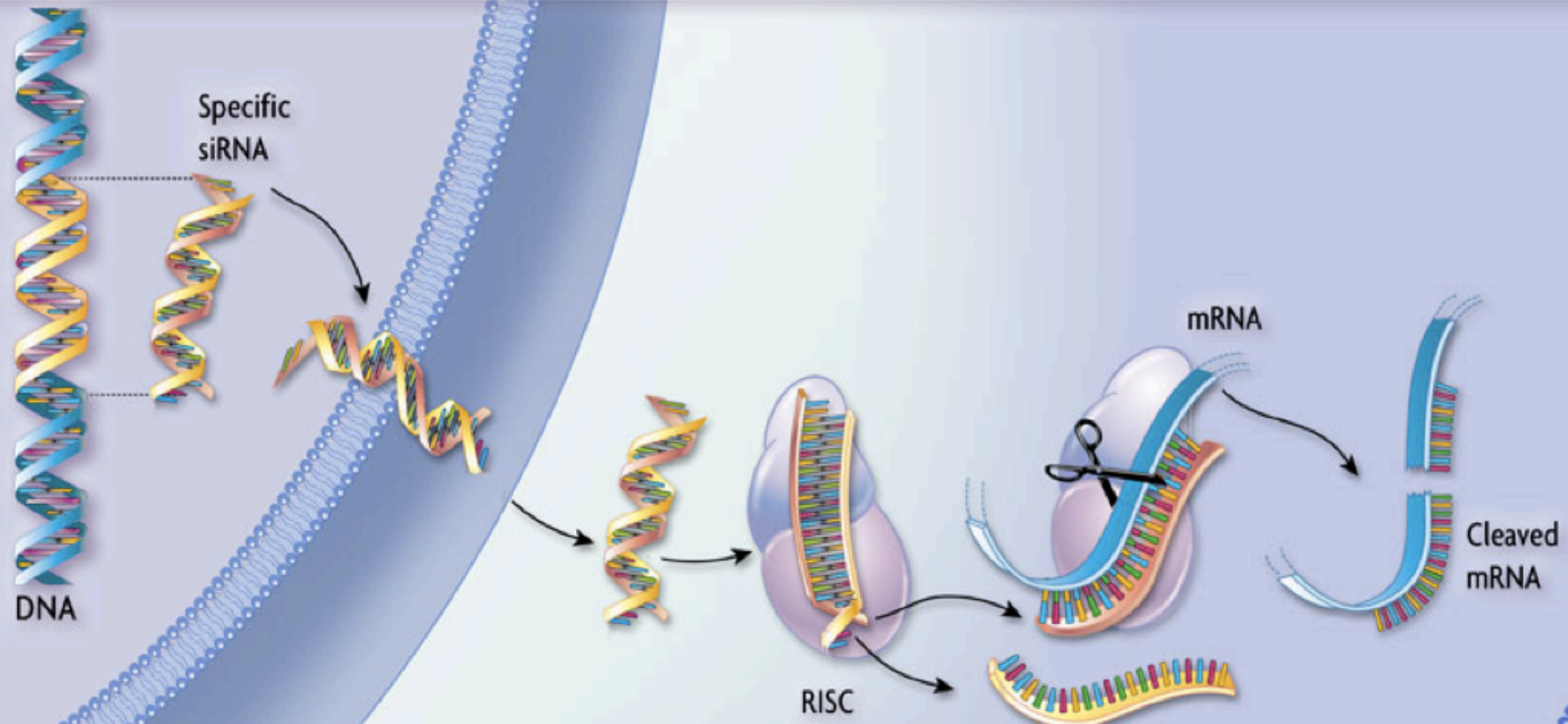
Concurrent R&D Strategy



DNA makes RNA makes Protein



The RNAi Natural Process



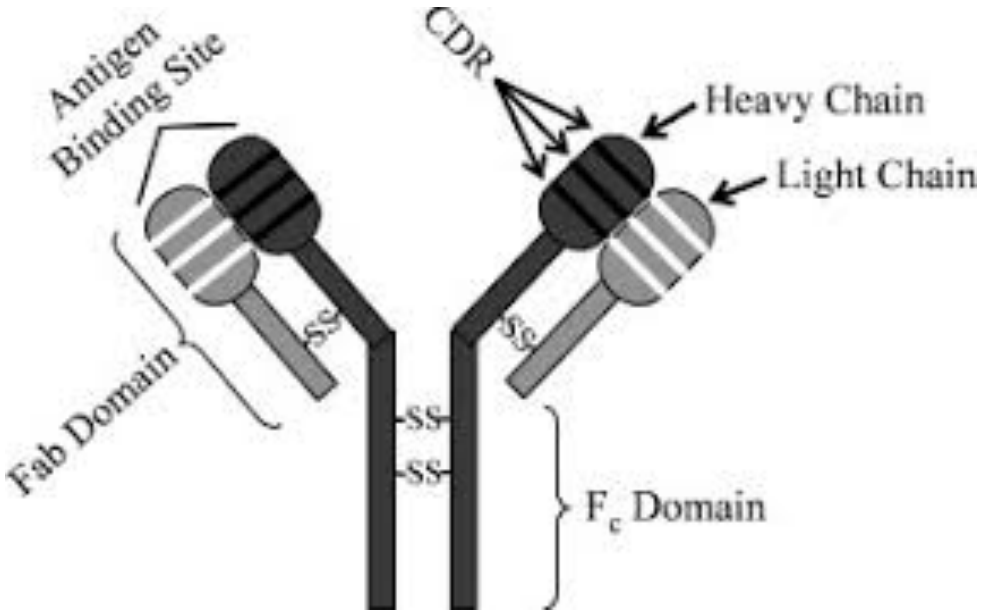
A Small interfering RNA (siRNA), a 21-25 base pai RNA strand, is targeted to a specific gene.

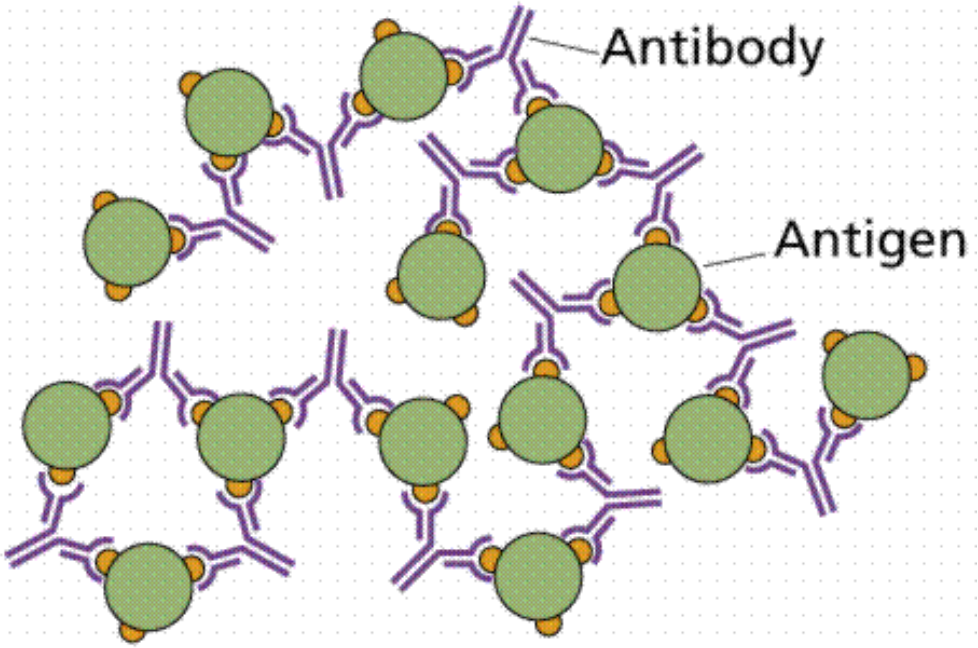
B Within cells, siRNA unwinds and is incorporated into RISC, a stable protein-RNA complex.

C siRNA is directed to a targeted messenger RNA (mRNA) that is known to be involved in a disease pathway.

D The mRNA undergoes degradation, thereby interrupting the protein synthesis of the targeted gene.

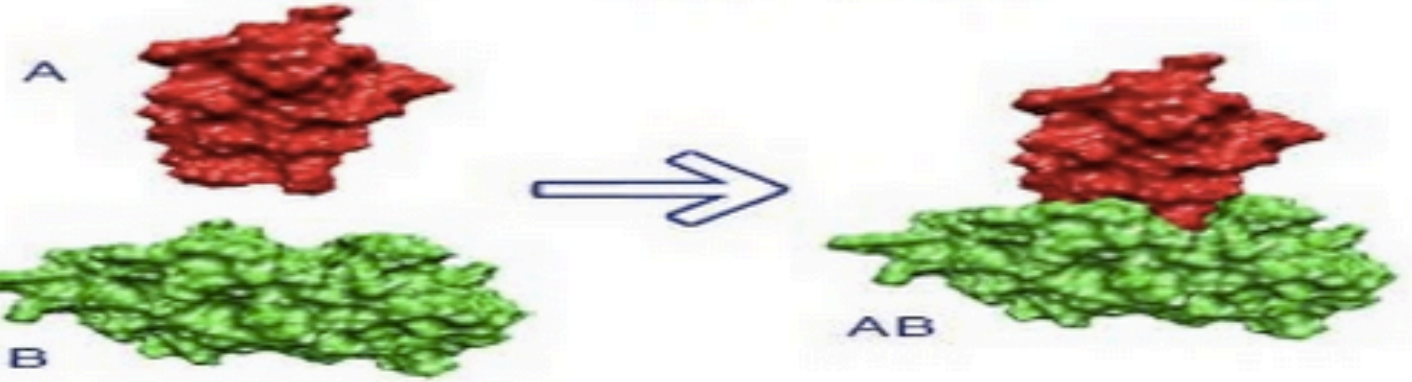
Antibody Structure/Function
scFV and Immunoprecipitation Tools



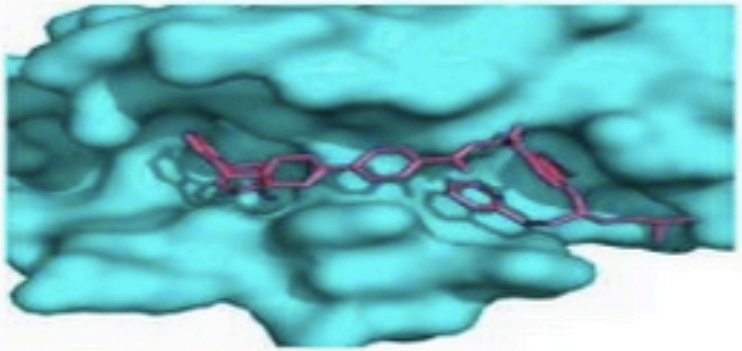


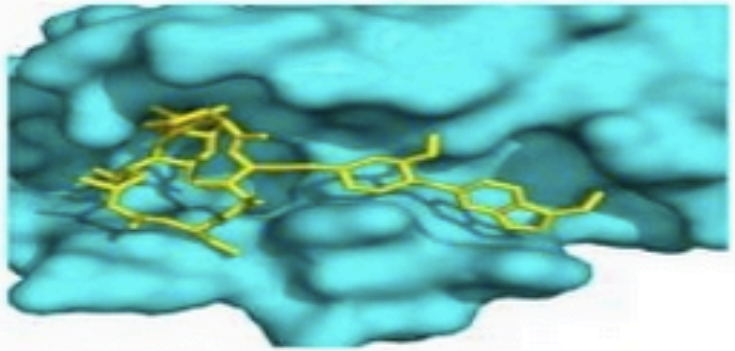
Molecular Interactions
Lock and Key – Hand in Glove

- Protein-Protein Docking



- Protein-Small Molecule Docking



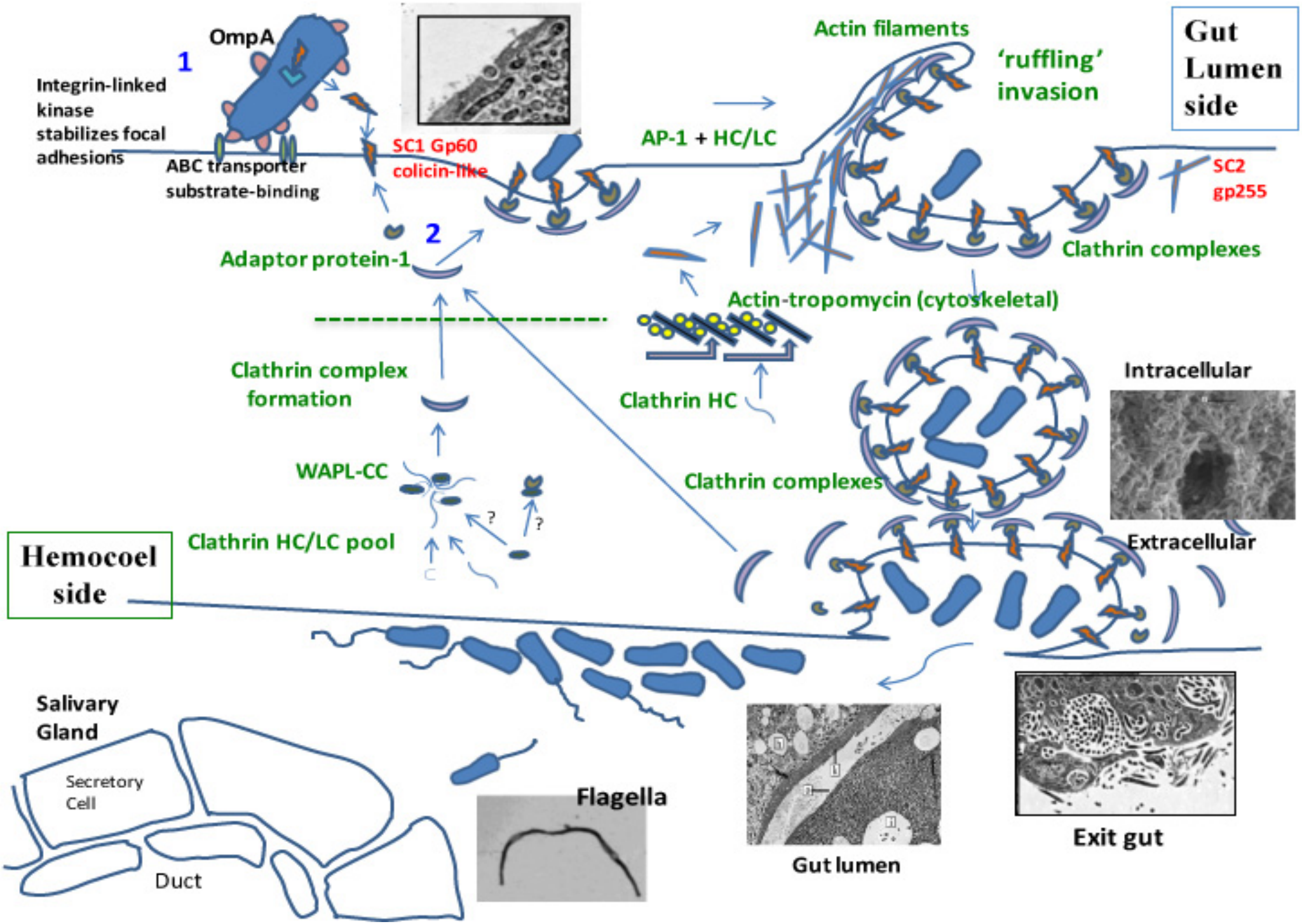


Effector–Genomics Approach: Judy Brown, U AZ

- Transcriptomics
  - Whole body, gut, salivary glands (adult) +/- Liberibacter infection
  - Whole body nymph +/- Liberibacter infection
  - mRNA expression profile, adults, stage 1/2, 3, 4/5 instars
- Proteomics
  - Whole body, gut, salivary gland proteins identified by mass spec
- Endosymbiont genomics from dissected ovaries
- Protein association studies with yeast two-hybrid system and co-immunoprecipitation
- RNAi effects
  - mRNA expression, mortality, reduction in transmission bioassays
  - Related Psyllid Shield concept from bioassays and field modeling

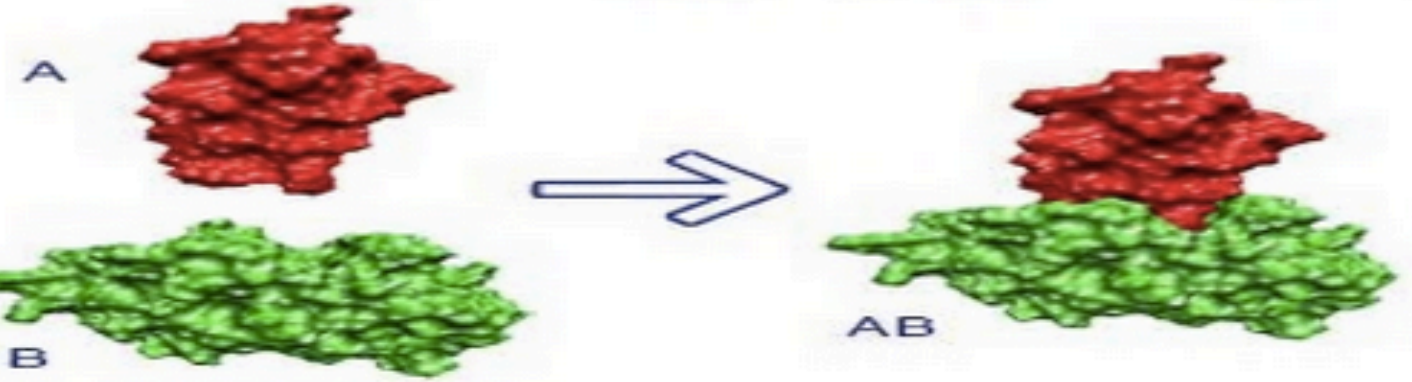
Genomics Approach: Discovery of New Actives

- Enormous, detailed bioinformatics database publically available as a foundation for future work
- A model for invasion in which CLas- and prophage-encoded effectors transform the endocytic host pathway into a ‘pathogen-mediated phagocytic scenario’ utilizing membrane ruffling in CLas interactions with the gut, leading to bacterial exit into the hemocoel and systemic invasion of other psyllid host tissues and organs
- Prioritized targets for RNAi knockdown
- Two candidate effectors whose knockdown resulted in >30% reduction in transmission, combination effects

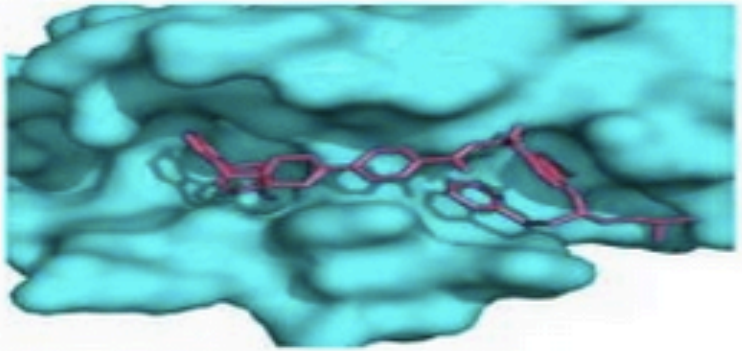

OmpA
1
Integrin-linked kinase stabilizes focal adhesions
ABC transporter substrate-binding
SC1 Gp60 colicin-like
Actin filaments
'ruffling' invasion
Gut Lumen side
AP-1 + HC/LC
SC2 gp255
Clathrin complexes
2
Adaptor protein-1
Actin-tropomycin (cytoskeletal)
Clathrin complex formation
Clathrin HC
Intracellular
WAPL-CC
Clathrin complexes
Clathrin HC/LC pool
Extracellular
Hemocoel side
Salivary Gland
Secretory Cell
Duct
Flagella
Gut lumen
Exit gut

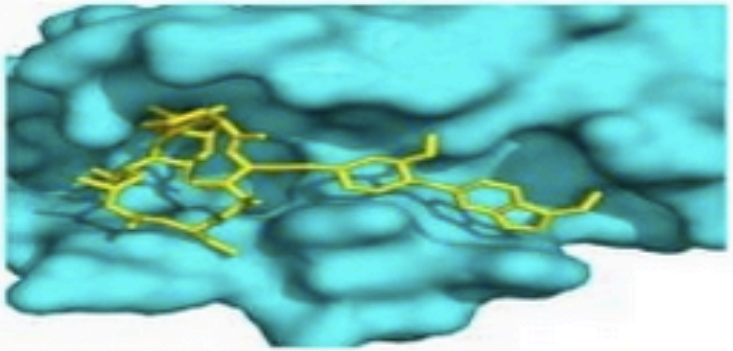
Molecular Interactions
Lock and Key – Hand in Glove

- Protein-Protein Docking



- Protein-Small Molecule Docking





Effector Mechanism–Library Approach:
R. Shatters, USDA, ARS

- Develop bioassays to challenge psyllid membranes
- Pursue artificial diet feeding for ways to block transmission
- Determine which combinations should be used to construct a synthetic gene that will be supplied to the driver team
- Testing single chain antibody fragments and fluorescent-tagged peptides for ability to block CLas-association with psyllid membranes

Library Approach: Discovery of New Actives

- 3 hexameric peptides that reduce psyllid nymphs ability to acquire/transmit *CLas* (AcTrans blockers)
- 2 bactericidal peptides that kill *CLas* in infected leaf tissue
- A combination of three AcTrans blocking peptides with one of the bactericidal peptides induced greater than 95% mortality in developing psyllid nymphs and none of the surviving nymphs have successfully acquired the CLas bacterium
- Product candidates: Peptides applied as synthetics, via CTV vectors or transgenics
- From antibody work: Two scFv fragments being evaluated in transgenics to inhibit efflux pump and reinstate apoptosis

Viral Driver System: Bryce Falk, UC Davis

- Collect ACP worldwide to detect naturally occurring viruses
- Generate sequence data and perform bioinformatics for virus identification
- Establish populations of candidate viruses in ACP
- Construct infectious virus cDNA's and use synthetic biology strategies for population suppression or replacement

Pipeline for small RNA deep sequencing and transcriptome profile analysis in ACP world populations

Collected *D. citri*: US (FL, TX, HI, CA) and many foreign locations (Taiwan, China, **Brazil** and Pakistan).

Generated small RNA and transcriptome libraries

Deep sequencing of small RNAs and transcriptomes for identifying viruses associated with *Diaphorina citri*

Sequencing

Bioinformatics analysis

Small RNA (HiSeq)

RNA-seq (HiSeq)

Confirm virus presence by (RT)PCR

Viruses Identified

Category	Family	Genus	Species	E-value	Population
dsRNA virus	*Reoviridae*	*Fijivirus*	*Nilaparvata lugens reovirus*	7e-70	CH, TW, FL, TX, HW
ssRNA virus	*Flaviviridae*	Unclassified	*Gentian Kobu-sho associated virus*	7e-56	CH, FL, HW
ssRNA virus	*Iflaviridae*	*Iflavirus*	*Deformed wing virus*	8e-112	**BR***, CH, TW
ssDNA virus	*Parvoviridae*	*Ambidensovirus*	*Mythimna loreyi densovirus*	1e-13	CH, TW, PK
ssRNA virus	*Bunyaviridae*	*Phasmavirus*	*Wuchang cockroach virus 1*	5e-40	CH, TW, FL
ssRNA virus	Unclassified	Unclassified	*Chronic bee paralysis virus*	9e-04	CH, CA, TX, FL
dsRNA virus	Unclassified phages	Unclassified	*WO prophage*	0.0	**BR***, CH, HW, FL, CA. TX

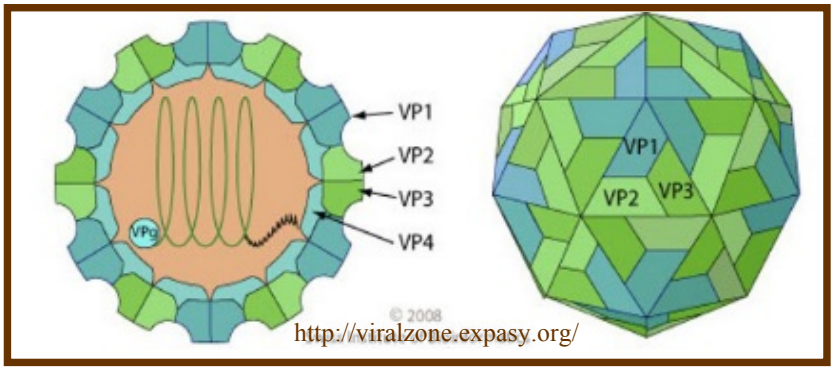
CH: China; TW: Taiwan; BR: Brazil; PK: Pakistan; FL: Florida; CA: California; TX: Texas; HW: Hawaii

Significant BLASTx hits to the viral database using contigs created from small RNA/RNA-seq libraries as query sequences

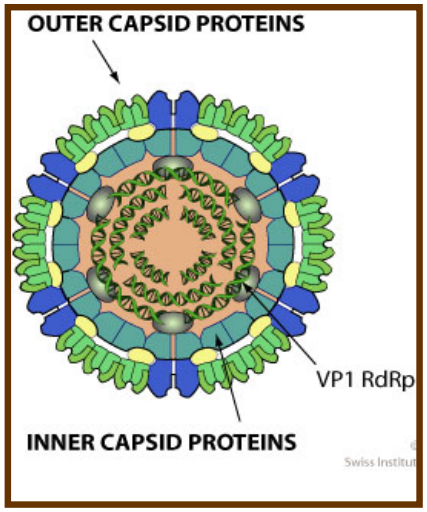
***Brazil samples only from laboratory *D. citri* colonies!**

Diaphorina citri reovirus (DcRV): dsRNA

Diaphorina citri picorna-like virus (DcPLV):+ssRNA



http://viralzone.expasy.org/



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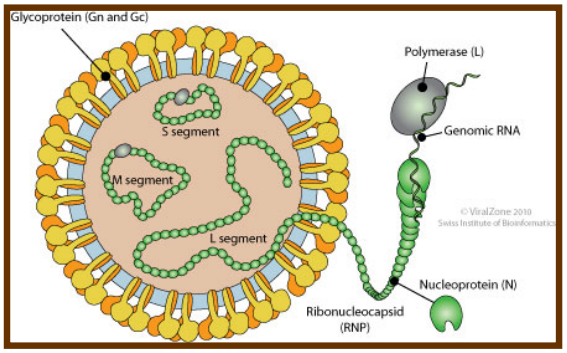
Diaphorina citri bunyavirus (DcBV): -ssRNA



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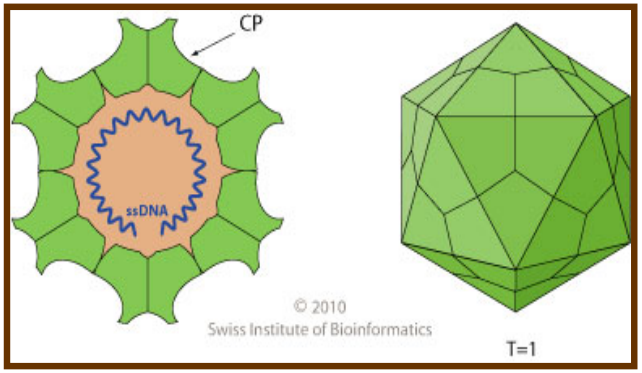
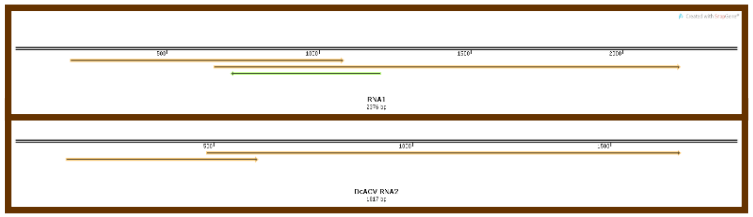
Diaphorina citri densovirus (DcDNV):ssDNA

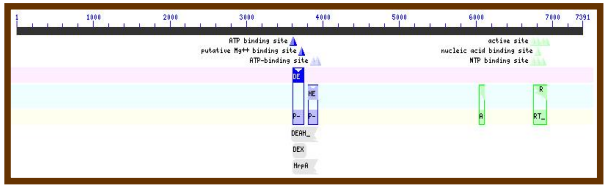


http://viralzone.expasy.org/

Diaphorina citri associated C virus (DcACV):+ssRNA



Diaphorina citri flavi-like virus: +ssRNA



D. citri virome

Nouri, et al. J. Virol, 90: 2434-45

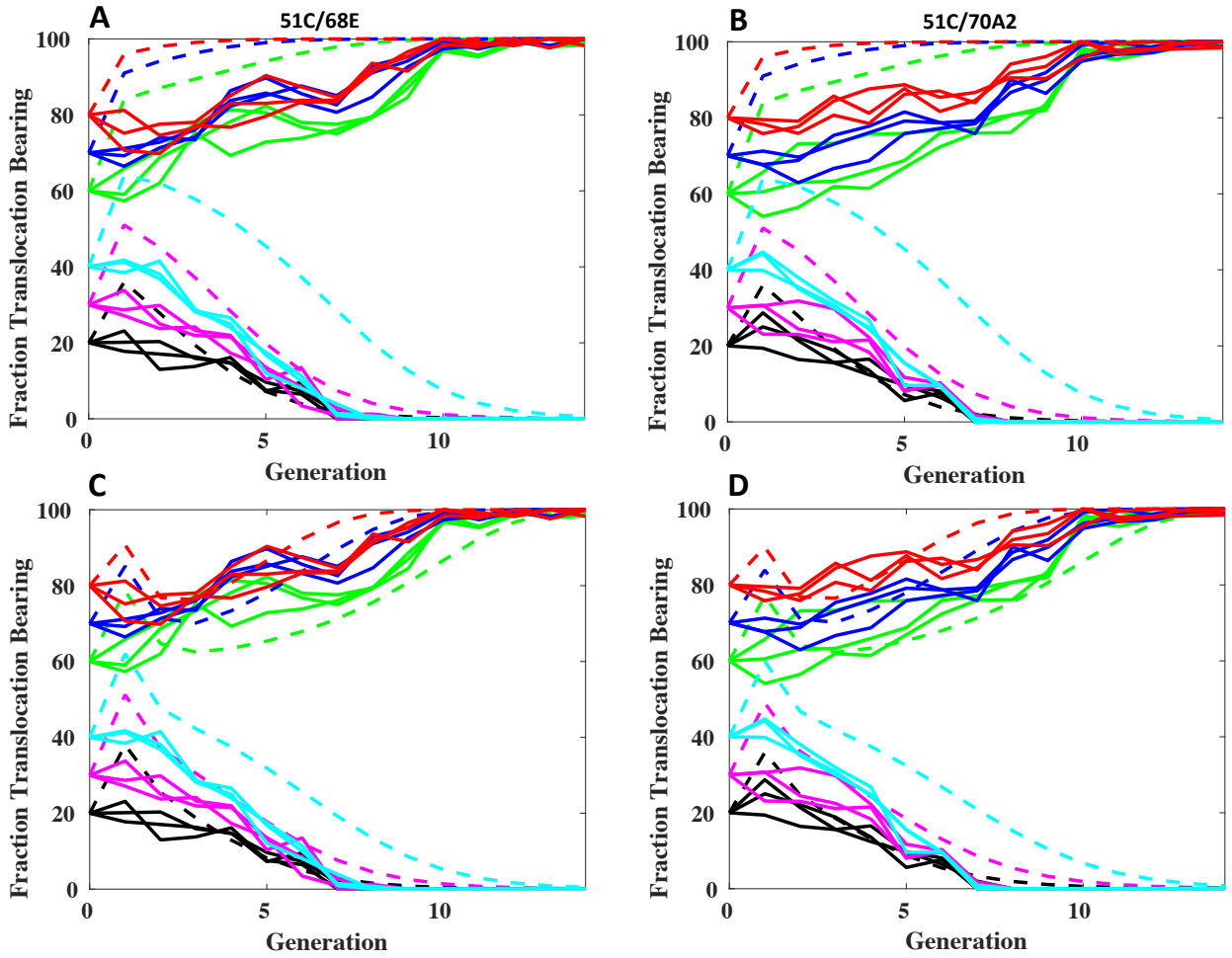
Bacterial Driver System: K. Pelz-Stelinski, UFL

- Survey of worldwide CLas collection from 11 countries and 2 US territories, all contain native Wolbachia with one of two different profiles
- Native Wolbachia does not competitively exclude CLas from wild populations but endosymbiont density is negatively correlated with CLas density
- Co-infections do not affect transmission of CLas
- Wolbachia-infected breeding lines have been obtained but it has not yet been possible to eliminate Wolbachia from cultures in order to introduce non-native Wolbachia
- From bioinformatics found to have a primitive immune system
- Cytoplasmic incompatibility shown to exist – driver basis if Wolbachia transformation is achieved

Chromosomal Driver System: Bruce Hay, Cal Tech

- High-threshold system
  - Local and reversible release based on underdominance
  - Reciprocal chromsome translocations create this phenotype
  - First demonstration of this system in any species created in this project using precision of modern synthetic biology tools
  - Improved tools, genetic system in place
- Low-threshold system
  - Cleavage drive, suitable for area-wide population replacement
- Synthetic biology reporter to induce cell death in response to a pathogen-dependent signal in the psyllid
  - Specific killing of infected individuals

Translocation-Based Population Replacement



Translocation-Based, High Threshold and Reversible Drive Achieved

- Requirements for proof of principle in Drosophila
  - transgene cassettes located on two different chromosomes,
  - a dominant marker created through the act of translocation,
  - a site-specific nuclease to create breakage within each cassette,
  - and unique sequences that can mediate recombination between the two chromosomes
- Generally applicable because of the common genetic behavior of reciprocal translocations in diverse species
- Because translocation-based drive is based on the behavior of entire recombinant chromosomes it is likely to be very species specific and stable

Psyllid Cell Cultures and Psyllid Transgenesis

- Psyllid cell cultures would be extremely valuable to have for a number of studies such as prioritizing effectors and optimizing drive components
  - Some success with other surrogates in Falk lab, unsuccessful attempts to date to create immortalized lines in Hay lab
- Major bottleneck is the ability to transform psyllids as well as other insect pests in general in addition to lab model systems
  - Traditional egg injection methods are not easily adapted to psyllids because of the size of the egg and the sensitivity to manipulation
  - Efforts continue to develop alternatives based on the injection of DNA and transfection reagent complexes into the adult germline (Hay lab)

Current Status of Psyllid Transgenesis

- To achieve efficient germ-line transformation of wild psyllid (Handler):
  - "new knowledge and methodologies were required relevant to ACP rearing and handling, including embryonic, nymphal and adult development, reproduction, mating behavior, individual, small and medium-scale rearing, determination of optimal plant hosts for egg collections (Swingle) and adult rearing (Murraya), egg collection and egg handling methods pre- and post-injection, nymphal stage rearing, adult rearing and small-scale mating, and tests for rearing on artificial medium, among others…"
- Early ACP embryos can be successfully micro-injected with DNA vectors with a reasonable level (~30%) of survival that results in nymphal hatching
  - Procedures established for removing newly laid eggs from fresh flush, washing them in a non-ionic detergent to prevent clumping, transferring individual eggs to double-stick tape to hold them in place for injection
  - Refining injection techniques for successful micro-injection with limited damage to the embryo, however nymphs die before successful transfer to flush to complete development
  - If the tape is powdered with potato starch and the tape with attached eggs is placed adjacent to or on fresh flush a few will survive to adulthood, less than 1%
  - New methods in progress, artificial media, microinjection of eggs on leaf, biolistics

Rear, Release, Monitor: Joseph Patt, USDA, ARS

- Identify and optimize mass-rearing procedures to be used in each state (CA, FL, TX)
- Use current knowledge of ACP auditory, olfactory, and visual attractants to optimize trapping procedures in preparation for release of nuPsyllid

Economic Modeling: Neil McRoberts, UC Davis

- Develop ACP phenology model and integrate with USPEST system housed at Oregon State University
- Develop population genetics sub-model to evaluate wild type ACP – nuPsyllid mixing
- Define economic analyses and integrate with social structure analysis
- Model and analyze social/economic impact of nuPsyllid and its efficacy

Public Outreach: Beth Grafton-Cardwell, UCR

- Organize state teams and develop messaging
- Meet with research team to understand research progress and developments in order to create educational materials
- Design web page, blogs, other social media
- Promote research findings at citrus industry events

Adoption of Drivers

- Step-by-step approach to research and public engagement
- Case by case combination of confinement and containment
- Environmental Assessments and Environmental Impact Statements under NEPA (National Environmental Protection Act) versus
- Ecological Risk Assessments allow for benefits and risks compared to alternatives and doing nothing
- Coordinated Framework for the Regulation of Biotechnology (USDA, EPA, FDA) no clear jurisdiction, currently FDA is default in a few cases
- International and trade implications

Phased Testing Pathway for Gene Drive

- Phase 0: Research Preparation
- Phase 1: Laboratory-Based Research
- Phase 2: Field-Based Research
- Phase 3: Staged Environmental Release
- Phase 4: Post-Release Surveillance

Science: Providing Solutions or More Problems?

Organizational Challenges

"Systems to share data and new knowledge will be needed as future gene-drive modified organisms are developed and prepared for release in confined field trials and into the environment."

Knowledge-Deficit Model

A one-way instruction of laypersons by experts with an expectation that public support will be the outcome.

"While information is important, and learning is important to forming opinions and decision making, research shows that it is not deterministic in the way that the deficit model assumes."

Engagement

"Seeking and facilitating the sharing and exchange of knowledge, perspectives, and preferences between or among groups who often have differences in expertise, power, and values."

Engagement Builds Trust
Responsible science must be something we do with the public not to the public

Thank-you

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