AGROTOOLS: A toolkit for *Agrobacterium* strain development to advance plant transformation

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Agrobacterium 2025 meeting — 16 Sept, 2025

Low rates of T-DNA delivery can be the main bottleneck for transformation in recalcitrant species

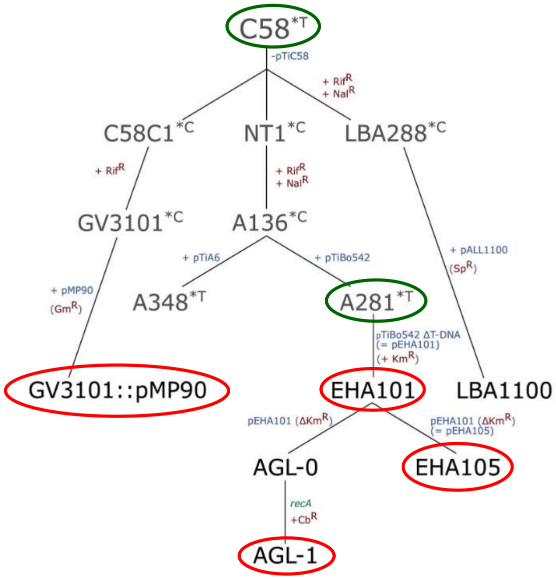
- The Strauss lab works on clonally propagated species/genotypes that are generally difficult to transform
- In some eucalypt and hop clones, efficient *in vitro* shoot regeneration can be achieved, but poor T-DNA delivery prevents regenerable tissue from making it through selection
- So we're exploring methods to potentially boost T-DNA transfer on the *Agrobacterium* side

EUCALYPTUS

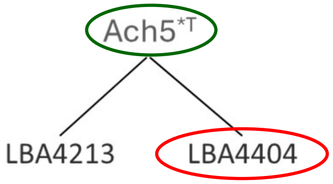
HOPS

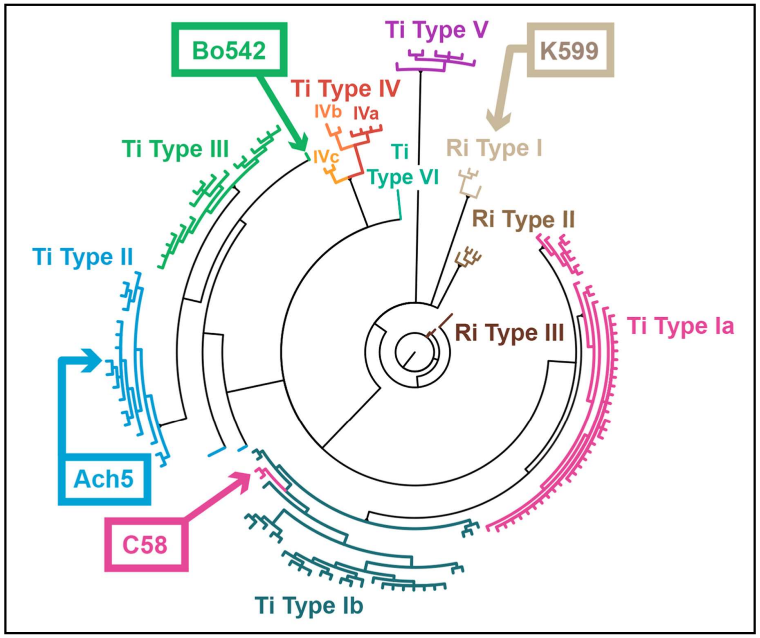
The conventional agro strain toolbox used for plant transformation is limited

- Strains used in the vast majority of stable plant transformation protocols are derived from a relatively narrow pool of wild-type progenitors
- Sampling from more diverse chromosomal backgrounds and virulence plasmid types could help identify strains better equipped to transform recalcitrant plants



Adapted from De Saeger *et al*. (2021)





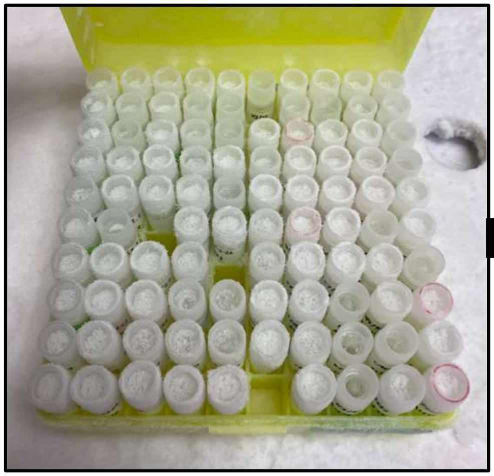
From Goralogia et al. (2025)

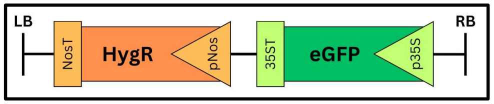
Reconstituted phylogenetic tree showing relationships between virulence plasmids of sequenced strains reported in Weisberg et al. (2020)

Strauss lab NSF project

Zak Heinhold
PhD student

Insert binary vector



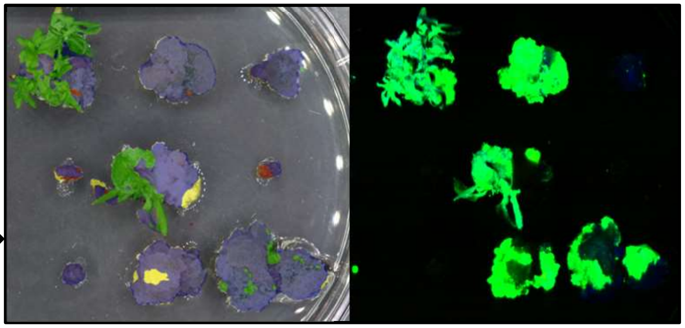


Transform plants

Modified Media

- No Acetosyringone
- Low Hygromycin (1mg/ml)
- No plant growth hormones
- 3 plates per strain

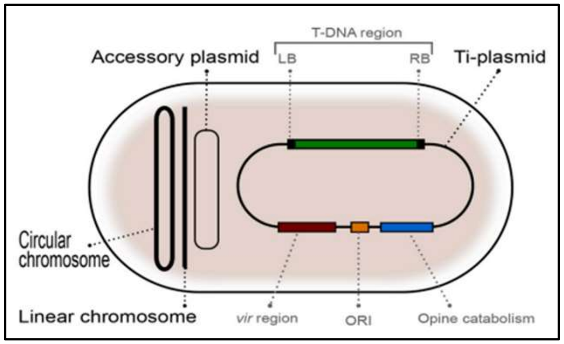
Phenomics Analysis



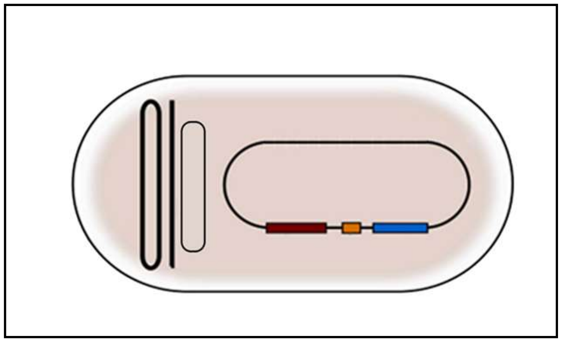
- Transgenic tissue
- Regeneration
- Necrosis

Disarmament is converting a wild-type strain to a plant transformation-ready one

- In addition to their two chromosomes and 0-6 accessory plasmids, pathogenic wild-type strains of *Agrobacterium* contain a virulence (Ti or Ri) plasmid
- The virulence plasmid carries both oncogenes (contained in the T-DNA region) and DNA transfer machinery (contained in the *vir* region)
- Disarmament refers to deleting the native T-DNA region from the virulence plasmid while retaining the *vir* region, leaving intact the strain’s natural ability to transmit DNA to plant cells



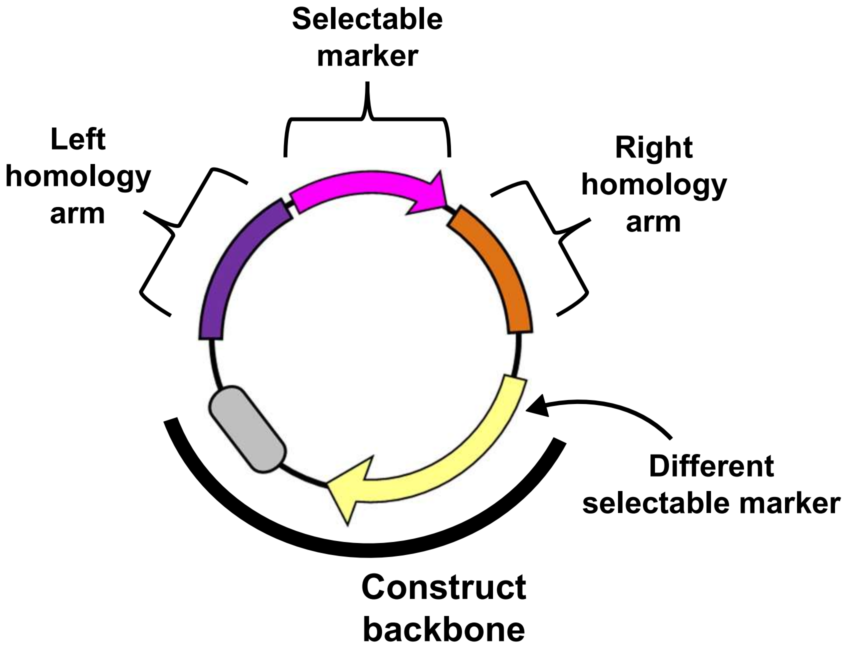
Wild-type *Agrobacterium* strain



Disarmed transformation strain

All *Agrobacterium* cell configuration schemes adapted from De Saeger *et al*. (2021)

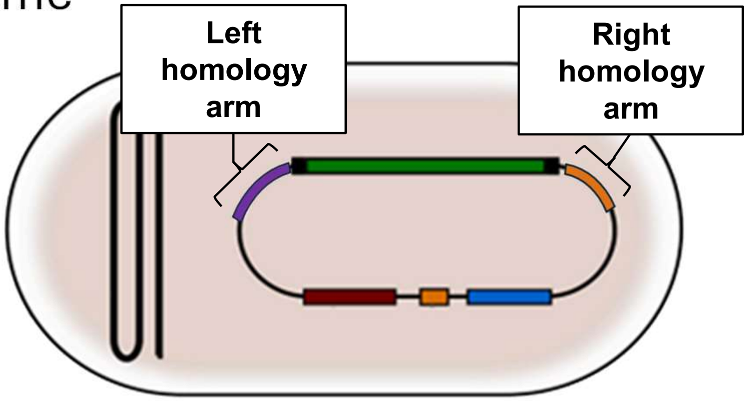
Double-crossover homologous recombination strategy for disarmament



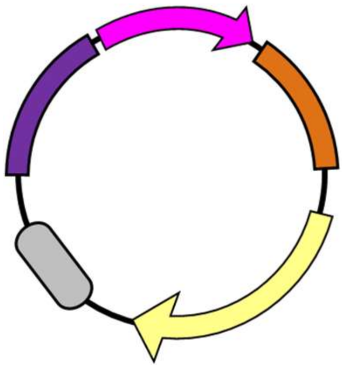
MINIMAL CONSTRUCT DESIGN

- This method utilizes an endogenous DNA repair pathway in bacteria (mediated by *recA*) to obtain colonies where recombination has occurred between two target sites, each with sequence homology to fragments in a cloned construct
- These recombination events are rare, but the use of selectable markers helps identify colonies with desired outcome

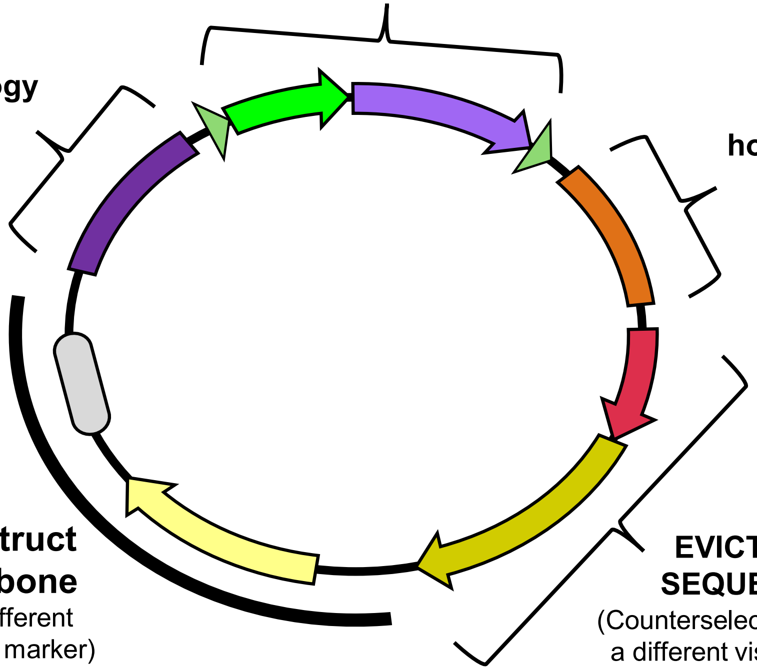
TARGET STRAIN



Advancing the design of disarmament constructs



MINIMAL CONSTRUCT



REPLACEMENT SEQUENCE
(Excisable selectable + visual markers)

Left homology arm

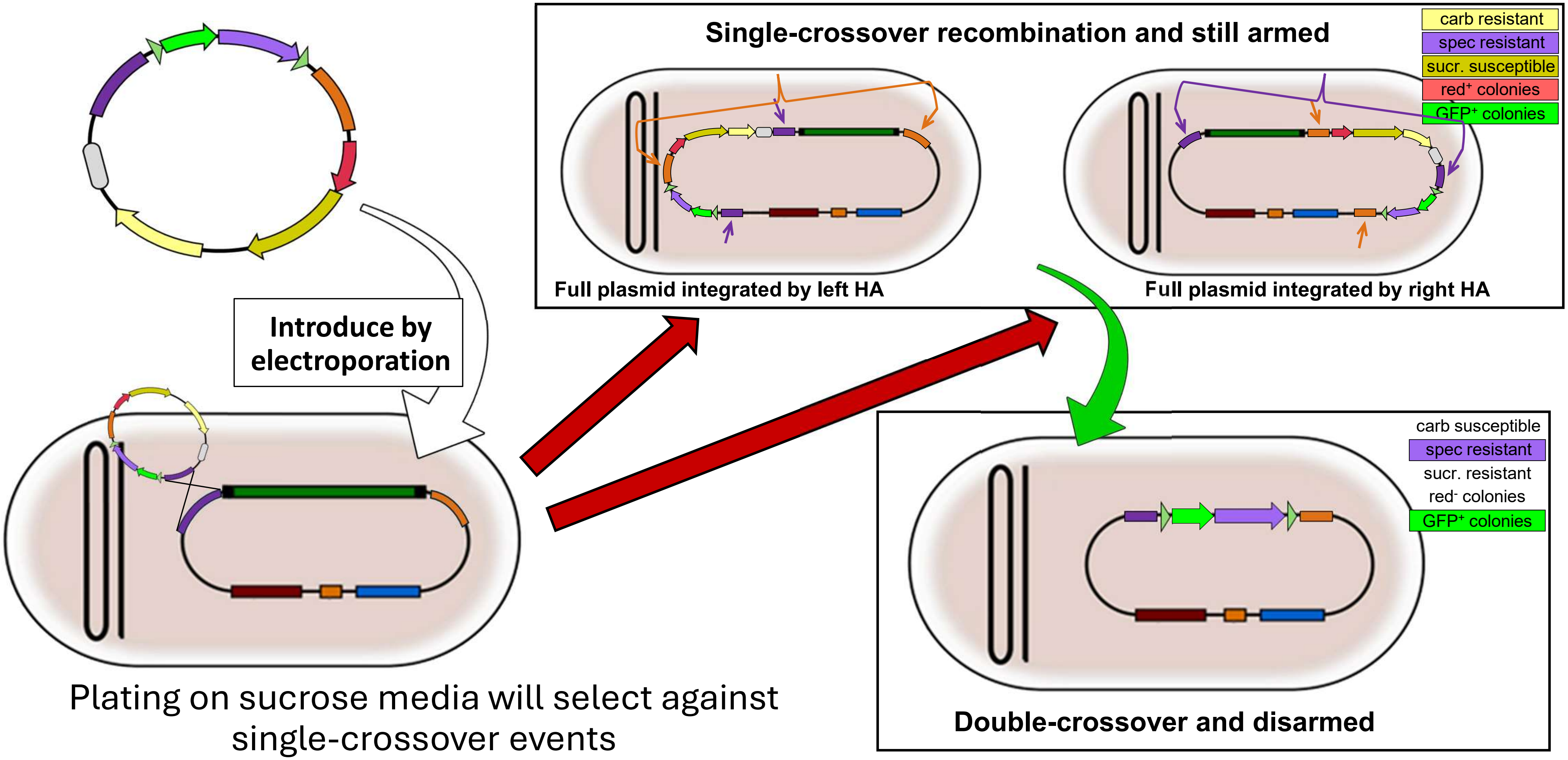
Right homology arm

Construct Backbone
(with different antibiotic marker)

EVICTION SEQUENCE
(Counterselection marker + a different visual marker)

VISUAL REPORTER DESIGN
(inspired by Pennetti et al., 2025)

Marker-assisted homologous recombination mechanism



Introduce by electroporation

Single-crossover recombination and still armed

carb resistant

spec resistant

sucr. susceptible

red^{+} colonies

GFP^{+} colonies

Full plasmid integrated by left HA

Full plasmid integrated by right HA

carb susceptible

spec resistant

sucr. resistant

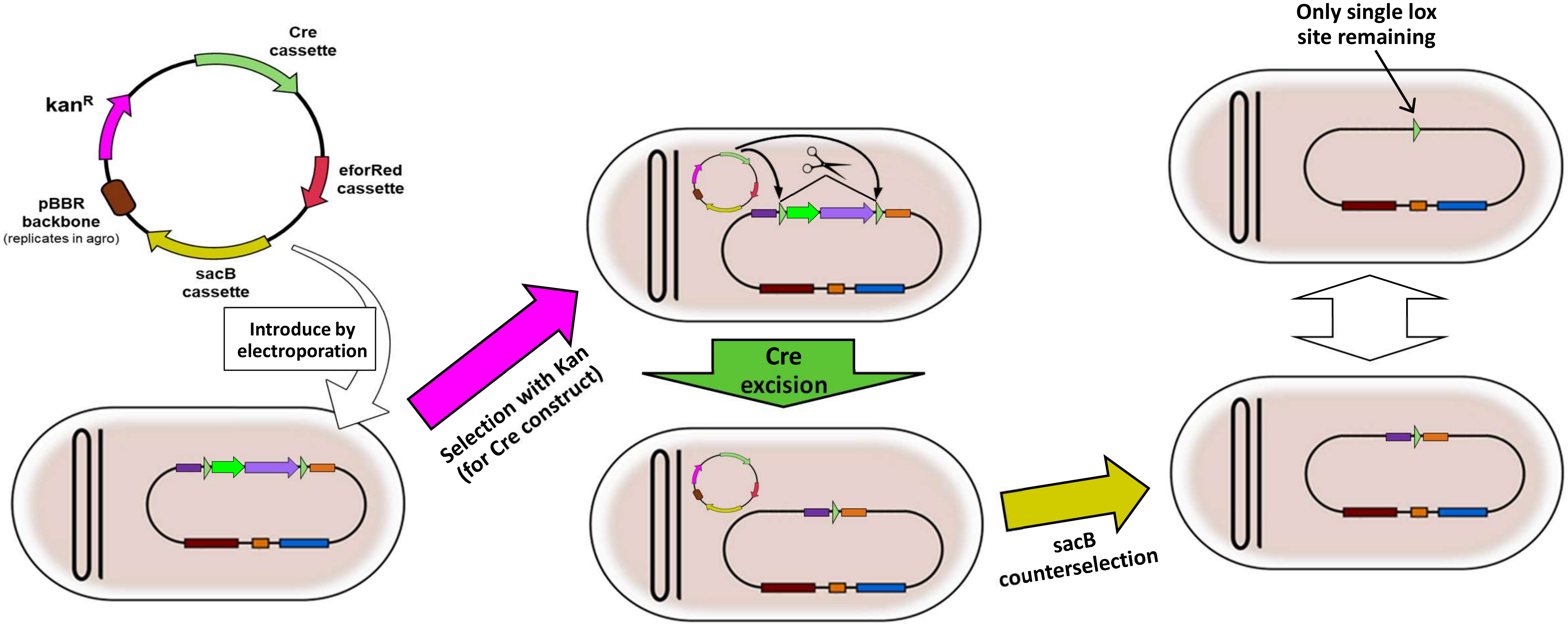
red^{-} colonies

GFP^{+} colonies

Double-crossover and disarmed

Plating on sucrose media will select against single-crossover events

Recombinase-mediated marker excision



Introduce by electroporation

Selection with Kan (for Cre construct)

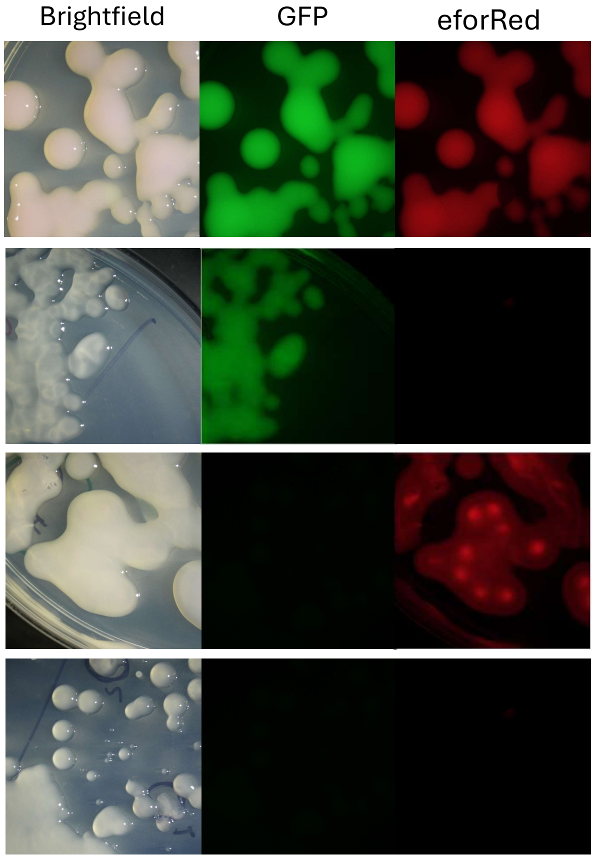
Cre excision

sacB counterselection

Only single lox site remaining

Visual reporters assist with tracking molecular configuration of colonies

Visual reporters assist with tracking molecular configuration of colonies

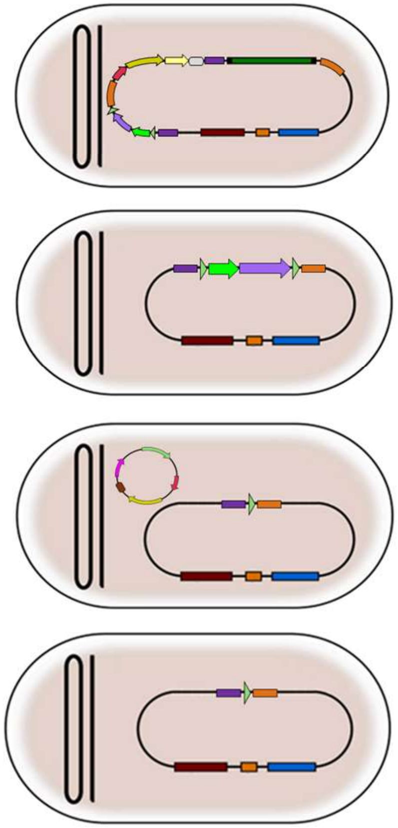


GFP and eforRed indicates a single-crossover event

GFP only indicates a double-crossover event

eforRed only indicates the presence of the Cre-recombinase containing construct

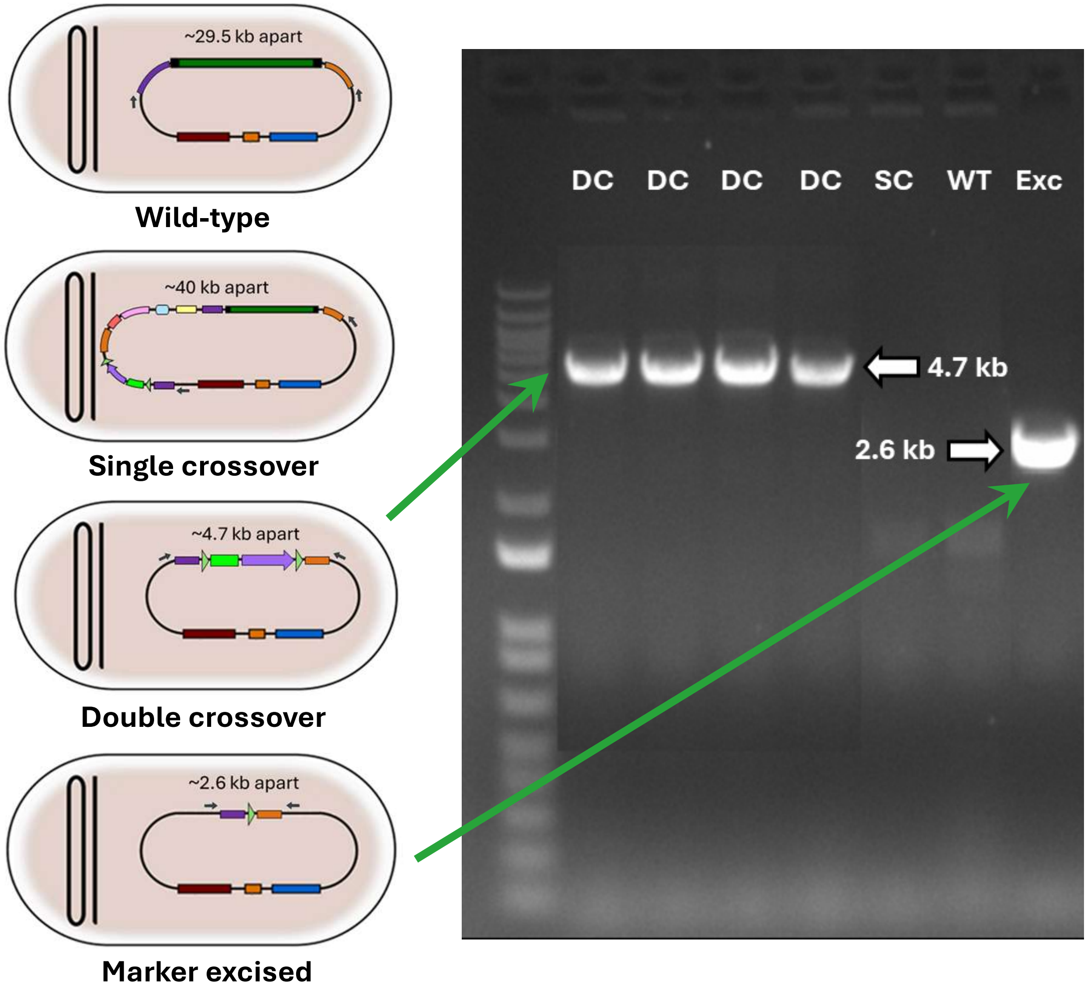
Expression of neither indicates removal of the Cre-recombinase containing construct



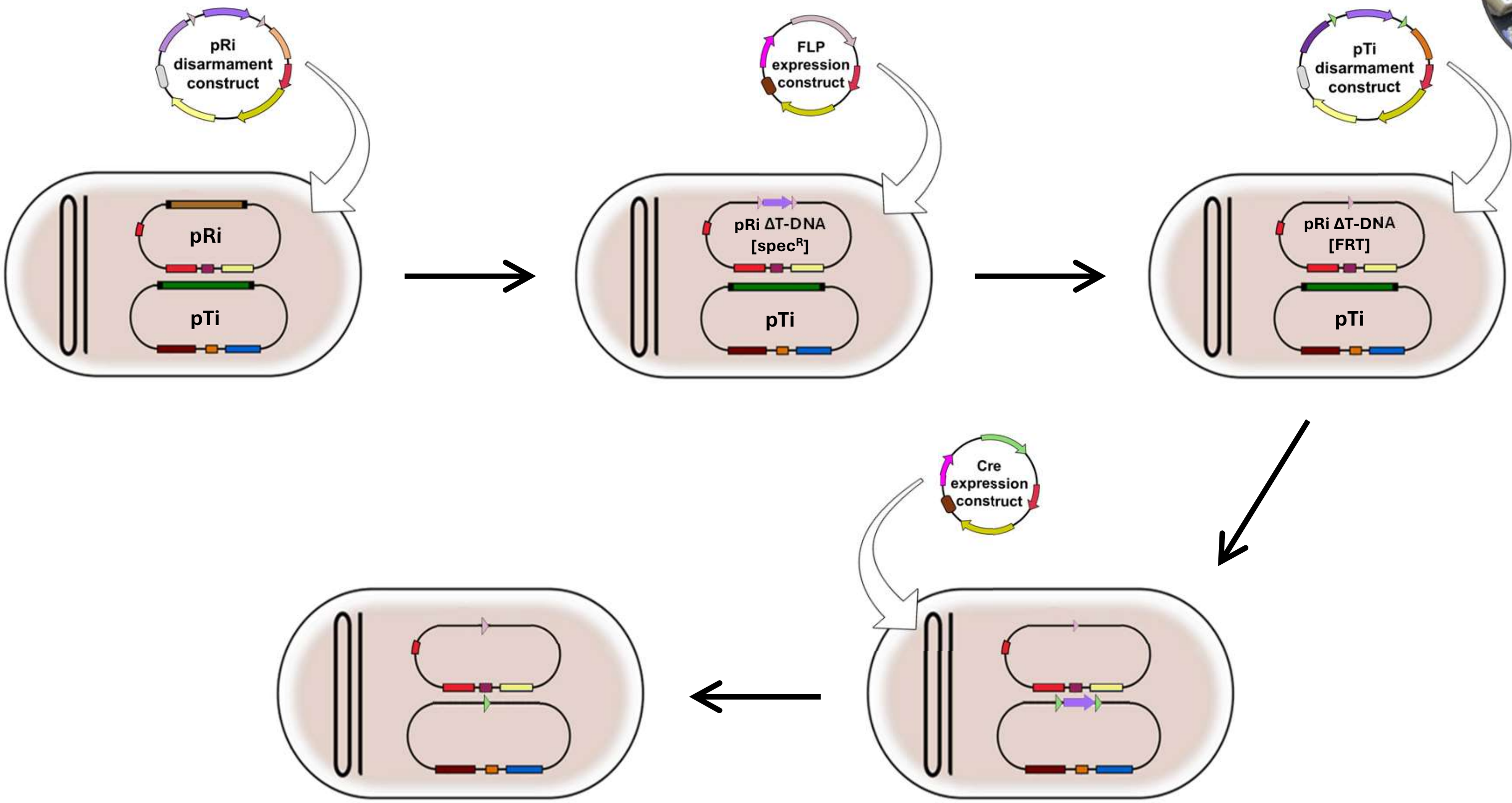
Using colony PCR to confirm double crossover and marker excision

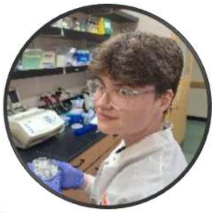
- Primers annealing to the target sequence just outside the left and right homology arms can be used to distinguish double crossover from both wild-type and single-crossover configurations

- Recombinase-mediated marker excision can also be confirmed using the same primer pair



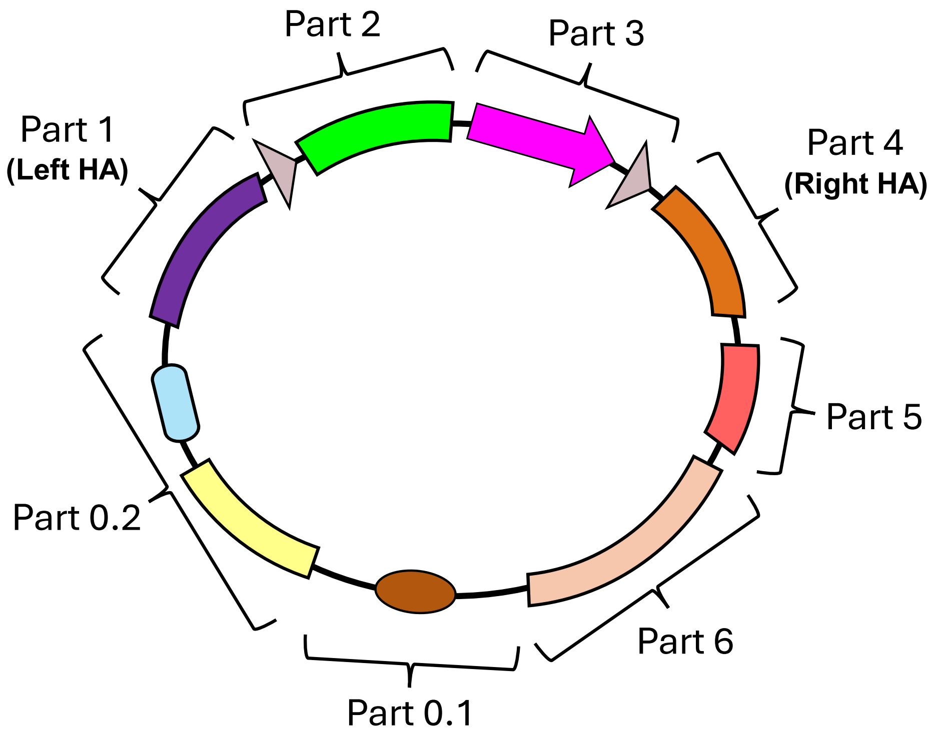
Double disarmament of wild-type strain Di1411 using this system





Jay Adams
Undergraduate researcher

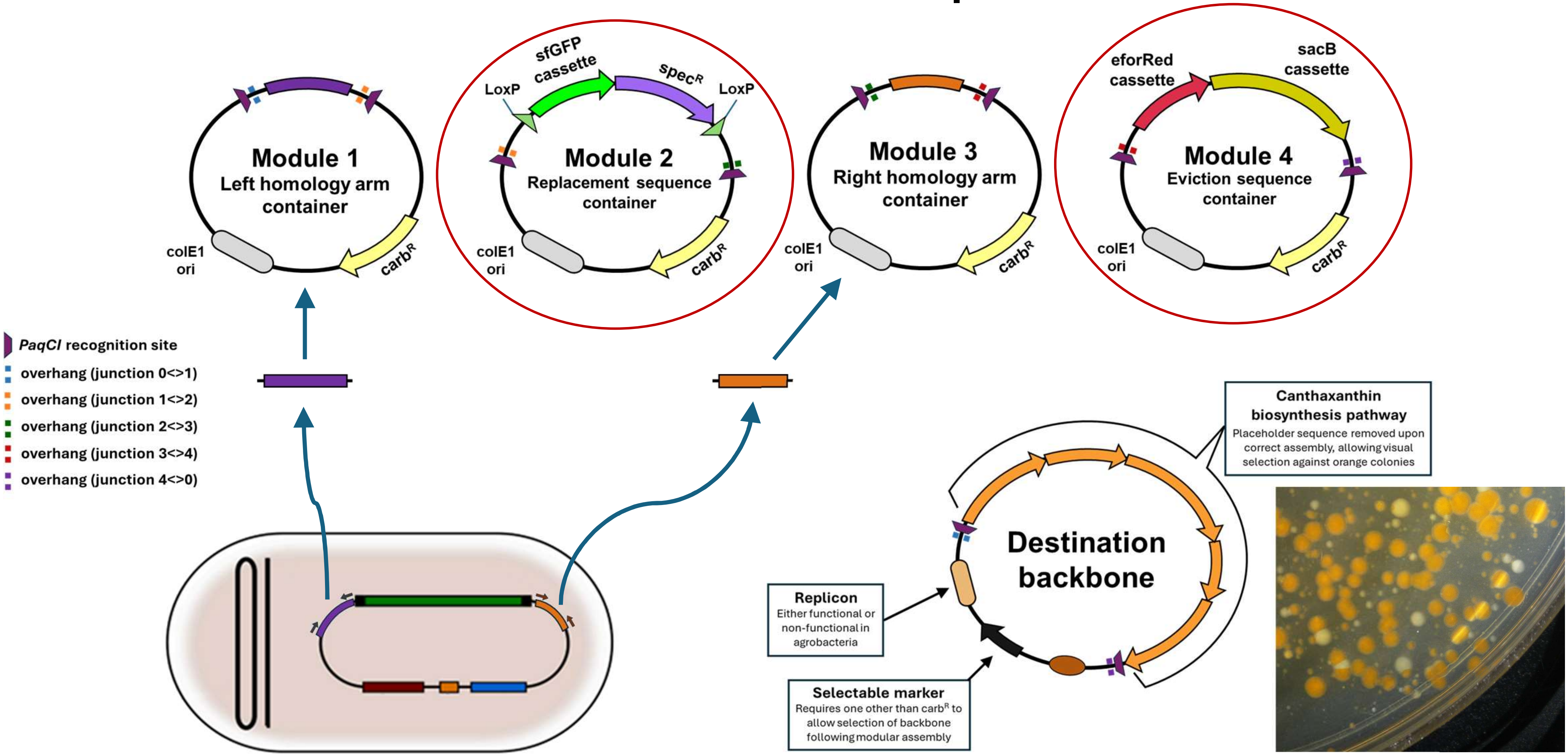
Reverse engineering our construct design



- The constructs we've used for disarmament so far have been assembled from 7-9 linear fragments amplified from disparate plasmid templates

- To streamline our *Agrobacterium* engineering plans we have built a rationally designed, versatile, and expandable cloning toolkit

Modular toolkit components

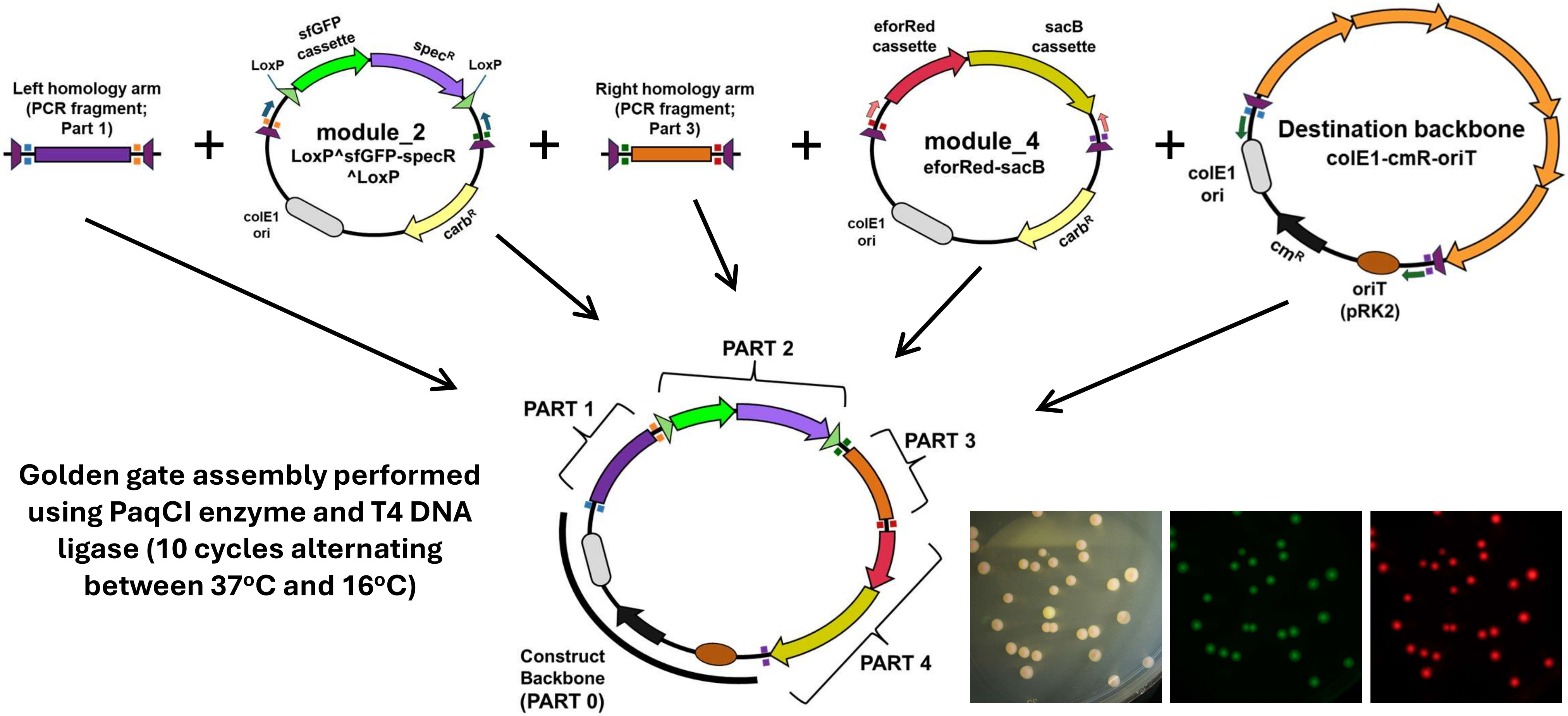


Canthaxanthin biosynthesis pathway
Placeholder sequence removed upon correct assembly, allowing visual selection against orange colonies

Replicon
Either functional or non-functional in agrobacteria

Selectable marker
Requires one other than carb^R to allow selection of backbone following modular assembly

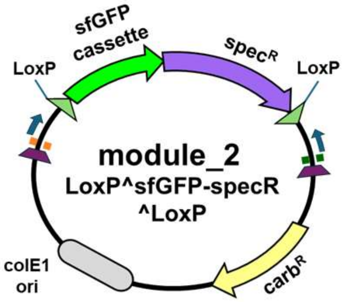
Assembly reaction

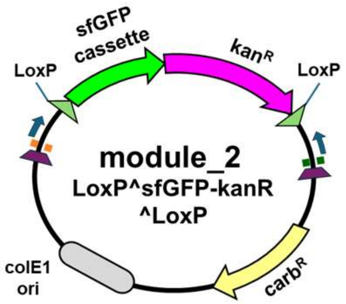


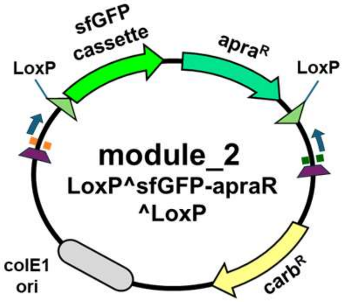
Golden gate assembly performed using PaqCI enzyme and T4 DNA ligase (10 cycles alternating between 37°C and 16°C)

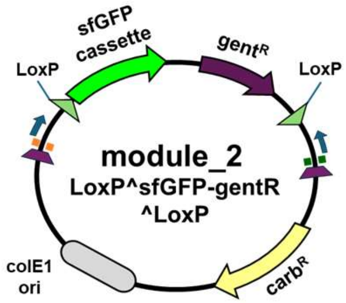
Variants and features of toolkit components

Options for deploying various antibiotic selectable markers



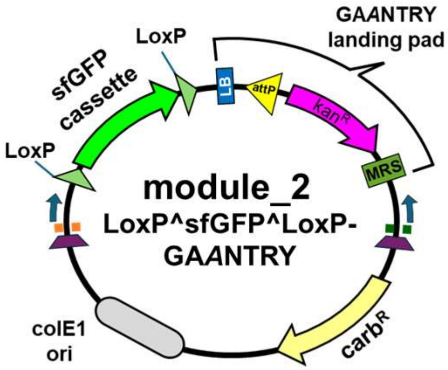






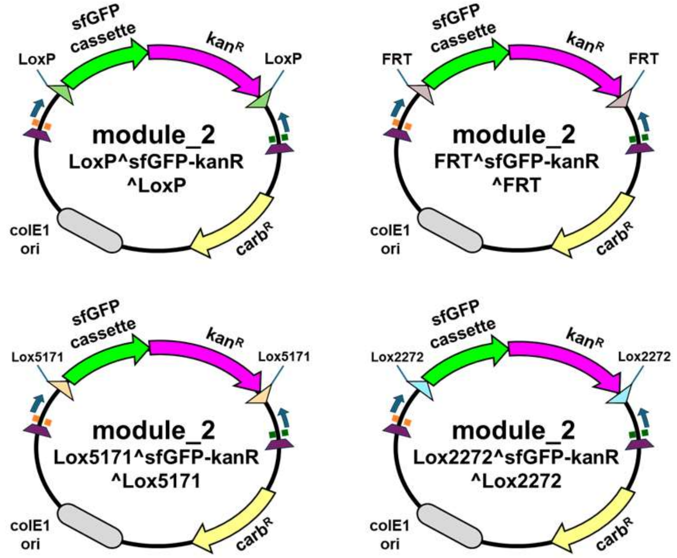
- Literature reports and our own observations have demonstrated variation between agro strains with respect to their basal sensitivity to different antibiotics

- Thus, we are including in the toolkit options for 4 different antibiotic selection cassettes



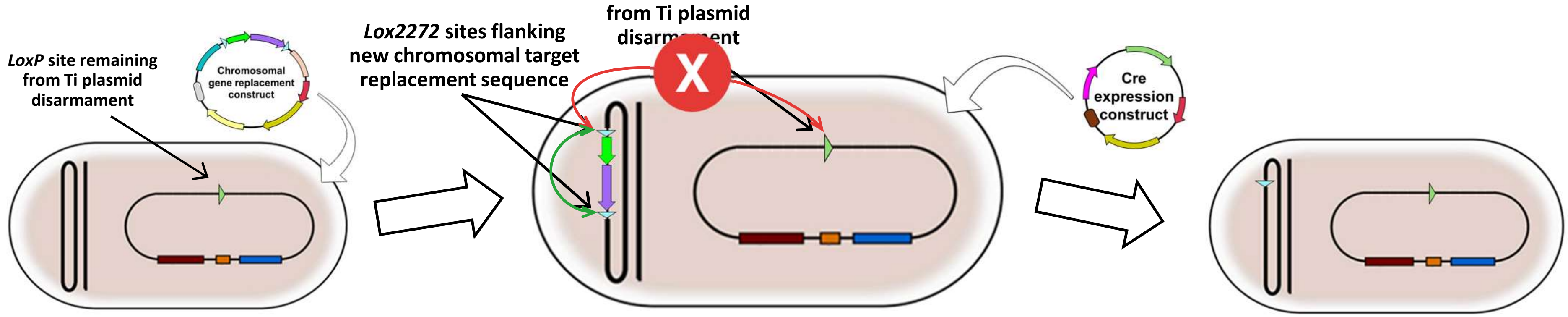
- Additionally, we designed a component for installing the “landing pad” for GA*A*NTRY cloning as strains are disarmed

Options for various recombinase excision sites flanking replacement sequence markers



- We designed variants of module 2 each with cargos flanked by one of four different recombinase sites which have been demonstrated not to be cross-reactive

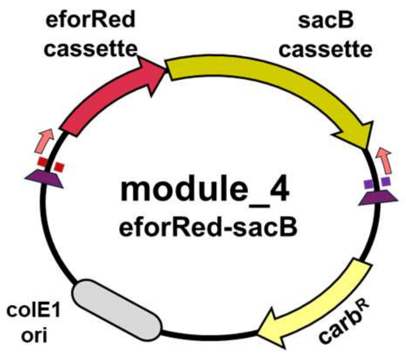
- This enables multiple consecutive modifications in the same strain background without risking undesired recombination with a “scar” remaining from a prior recombinase excision


LoxP site remaining from Ti plasmid disarmament

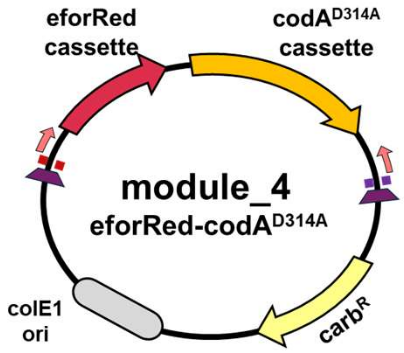
Lox2272 sites flanking new chromosomal target replacement sequence

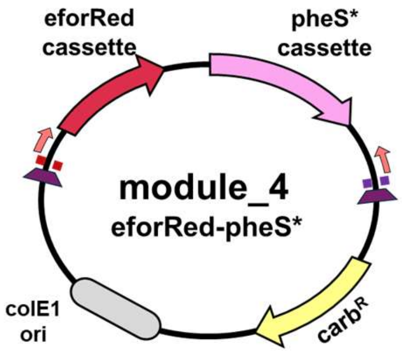
LoxP site remaining from Ti plasmid disarmament

Different counterselection markers



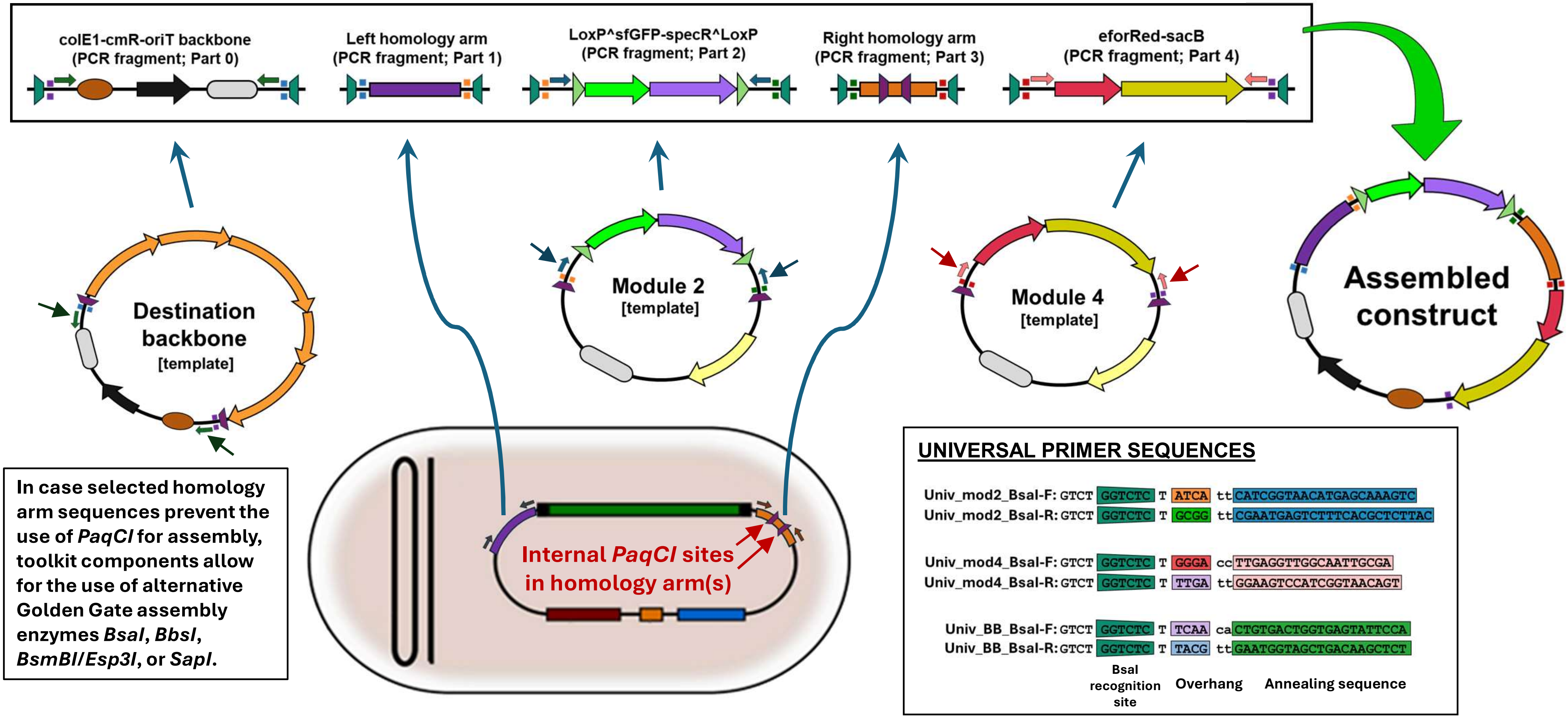
- sacB doesn't provide "tight" counterselection and has a propensity to mutate, resulting in loss of selective function





- We've synthesized expression cassettes for additional counterselection markers ($codA^{D314A}$ and pheS*) reported in other systems and are working on evaluating them in agro vs. sacB

Alternative PCR-based assembly strategy



In case selected homology arm sequences prevent the use of *PaqCI* for assembly, toolkit components allow for the use of alternative Golden Gate assembly enzymes *BsaI*, *BbsI*, *BsmBI*/*Esp3I*, or *SapI*.

UNIVERSAL PRIMER SEQUENCES

Univ_mod2_BsaI-F: GTCT GGTCTC T ATCA tt CATCGGTAACATGAGCAAAGTC
Univ_mod2_BsaI-R: GTCT GGTCTC T GCGG tt CGAATGAGTCTTTCACGCTCTTAC

Univ_mod4_BsaI-F: GTCT GGTCTC T GGGA cc TTGAGGTTGGCAATTGCGA
Univ_mod4_BsaI-R: GTCT GGTCTC T TTGA tt GGAAGTCCATCGGTAACAGT

Univ_BB_BsaI-F: GTCT GGTCTC T TCAA ca CTGTGACTGGTGAGTATTCCA
Univ_BB_BsaI-R: GTCT GGTCTC T TACG tt GAATGGTAGCTGACAAGCTCT

BsaI recognition site **Overhang** **Annealing sequence**

Summary

- We are building a molecular cloning toolkit for *Agrobacterium* strain modification with the aim of it being user-friendly, versatile, and expandible
- Intended to be used for both generating novel strains for plant transformation and improving existing ones
- Could serve as a resource for basic genetics studies in *Agrobacterium*
- These tools are likely functional in other plant pathogenic bacteria (*e.g.*, *Pseudomonas* spp.) and could likewise be used for genetic studies in those species

Acknowledgements

Contributors:

- Greg Goralogia
- Zak Heinhold
- Bethani Sutliff
- Jay Adams
- Tanner Whiting
- James Warner
- Victoria Conrad
- Steve Strauss

Other Strauss lab members:

- Veerendra Sharma
- Cathleen Ma
- Kate Permyslova
- Anthony Marroquin
- Carter Mucken
- Sydney Gould
- Dana Howe
- Parker Wheeler
- Colette Richter

Oregon State University

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