Reprogramming Microbes to Silence or Cater to their Hosts:

Beta-Carotene Production and RNAi Delivery

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Humans contain billions of microbes which help digest our food and produce vitamins to suppliment our diet, while plants such as corn harbour microbes within their tissues which can extend the metabolic capacity of their host. In an attempt to exploit these patterns of microbial habitation, we built biobricks for beta carotene production using the broad host range plasmid pDSK-GFPuv for bacterial expression of either carotenoid metabolic genes belonging to the soil microbe *Erwinia uredovora*, or for RNAi targeted expression silencing of the corn TB1 gene. These plasmids were electroporated into either the human probiotic *Escherichia coli* Nissle 1917 or corn endophytic *Klebsiella pneumonii* 342 and cultured within anaerobic jars mimiking a human intestinal tract or within living corn plants. While the synthetic beta carotene operon was found to work to produce visible amounts of beta carotene in the high copy pSB1A2 plasmid in lab strains of *E. coli*, it was not observed to result in significant production when inserted into the pDSK-GFPuv plasmid. Corn gene silencing may have occurred after injection with RNAi containing Klebsiella pneumonii 342, plants did not yet display the phenotype at the time of writing.

Beta-carotene Production

Roughly 400 million people worldwide are at risk of vitamin A deficiency, of which 100-200 million are children (1). Deficiency can lead to progressive blindness and death and is preventable with a healthy diet containing significant plant derived beta-carotene pigments which are readily converted in the body to vitamin A. For many socio-economic and environmental reasons which are further explored by the iGEM 2008 Calgary Ethics team on their website (1) , improved diet is not a likely treatment for everyone. Knowing this, we propose a synthetic biology answer to this world health problem: to create a plug and play beta carotene production program in a broad host range plasmid to reprogram human and corn associated bacteria to produce this vitamin where it was not synthesized before. For transgenic intestinal vitamin production, we targeted the probiotic *E. coli* strain Nissle 1917 and several species of lactobaciilli which might be ingested in locally made yoghurts. A corn endosymbiotic bacteria, *Klebsiella pnumoniae* 342 was also picked for testing vitamin production inside a different biological system.

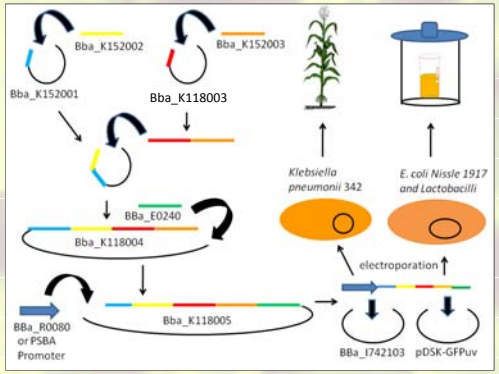

Figure 1: Methods used to build and test a synthetic operon for beta-carotene production.

Results: Beta-carotene

We obtained *Erwinia uredovora* genes for beta carotene synthesis from Ethan Johnson of iGEM Minnesota and these were modified by PCR mutagenesis to conform to Biobrick standards before submission to the registry. These Biobricks were then employed in the construction of a synthetic operon by serial suffix addition cutting the host plasmid with SpeI and PstI and ligating in the next XbaI and PstI cut fragment (see figure 1). To provide a transcriptional terminator and a visual marker for complete operon transcription, we included the GFP Biobrick Bba_E0240 as the fifth gene in the composite device. Two different promoters were inserted to drive operon expression: the chloroplast 16S ribosomal promoter (PSBA) from a herbicide resistant Amaranthus plant (2) and the arabinsoe inducible promoter BBa_R0080. The complete transcriptional units housed in the high copy plasmid pSB1A2 did indeed result GFP signal emission and beta-carotene accumulation in the *E. coli* lab strain DH5α (figure) but when transferred to the low copy broad host range plasmid pDSK-GFPuv (2) neither fluorescence nor carotenoid production was observed (results not shown). These lab strains containing the synthetic operon were tested for survival and beta carotene production in a crude intestinal model consisting of fecal samples incubated at 37 degrees for 48 hours in anaerobic containers containing 200 mL of fluid universal media (figure 3A). No beta carotened production was detected in any jar, however *E. coli* Nissle tagged wtih pDKS-GFPuv did survive and maintain the plasmid in this environment (figure 3B).

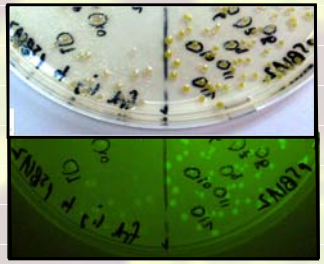

Figure 2: Visual confirmation of synthetic operon function on 1% arabinose plates. On the left are cells containing Bba_K118005, while those on the right contain the same Biobrick with the arabinose inducible promoter prefix. Similar results were obtained with the PSBA promoter.

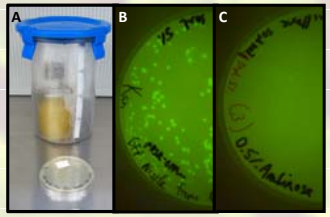

Figure 3: Testing beta carotene producing *E. coli* in an anaerobic inestinal model. 1 mL of OD600=0.8 cells were inoculated in 200 mL of fecal culture in fluid universal media and incubated for 48 hrs at 37ºC in anaerobic jars (A) . Speading 50 ul of culture on antibiotic plates showed *E. coli* Nissle containing pDKS-GFPuv survived (B) but pSB1A2 containing DH5α did not.

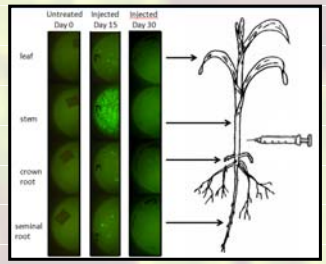

Figure 4: Testing endophyte behaviour and plasmid maintenance in corn. Plants recieved two 10 µl injections of pDSK-GFPuv containing *Klebsiella pneumoniae* 342. 500 mg samples were taken, macerated , resuspended in 500 µl phosphate buffer and spread on kanamycin plates for CFU counts.

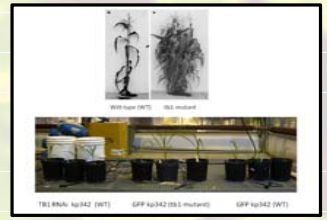

Figure 5: Testing kp342 potential for TB1 silencing. Endophytes contained pDSK-GFPuv modified to contain and express Bba_K152010. Plants received two 10 µl injections and were evaluated for at two weeks age for increased branching relative to pDSK-GFPuv Kp342 injected tb1 mutants (middle) and pDSK-GFPuv injected wild type (right). (3)

Discussion

We attempted to build plug and play plasmids which could be easily transferred to different bacterial species to co-opt their growth habits for human benefit. We were able to boot up a beta carotene production function in the lab strains of *E. coli* Dh5α when expressed off of the high copy plasmid pSB1A2 and supplimented with exogenous arabinonse (figure 2), but this effect was not observed using the low copy plasmid pDSK-GFPuv. When put into the intestinal model however these lab strains of *E. coli* were not able to survive or produce beta carotene after only two days, while pDSK-GFPuv in the probiotic strain Nissle 1917 was observed to survive and express GFP. Clearly further work will need to be done to adapt the synthetic operon for beta-carotene production from low copy plasmids and in biologically relevant microbial species. The TB1 RNAi construct expressed in pDSK-GFPuv was successfully created, electroporated into kp342, and injected into corn plants, but as the silencing phenotype manifests itself at later stages of plant maturity, it is not possible to comment on effectiveness or necessary additions to the strategy yet. BIGS will hopefully be promoted as a cheap, quick, and stable for studies in plant functional genomics worldwide.

References

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Thank You!

The University of Guelph's Alumni and Friends, Student Life, and the Gordon Nixon Leadership Award

Bacterial Induced Gene Silencing (BIGS) by RNAi

Double stranded RNA is recognized by eukaryotes as a signal for post transcriptional gene silencing and is so significant a discovery that it received the 2006 Nobel prize for Physiology or Medicine. It has gained importance in plant science as a tool for functional genomics to study gene function by mutant phenotype analysis, however in plants like corn, transgenic plant production takes up to a year. Viral Induced gene silencing (VIGS) is a quick and transient alternative in plants where it has been developed, however it has transient effects and does not result in stable phenotypes. As a quick and stable alternative, we propose to deliver RNAi signals into plants using plasmid expression in endophytes; a strategy similar to transkingdom RNAi developed for mice using *E. coli* (4). Since bacteria like *Klebsiella pneumonii* 342 can colonize corn plants in large numbers (5), we believe that constitutive RNAi expression in these cells as they die and lyse within the plant host, will result in dsRNA seepage into the plant resulting in systemic silencing of the targeted gene. We propose to call this technique BIGS (Bacterial Induced Gene Silencing). We designed a BIGS construction tool, which will accept any existing Biobrick and allow its orientation in an RNAi orientation (fragment in the sense orientation followed by a copy in the antisense orientation). To test our device, we targeted the corn TB1 gene for silencing which should result in a distinctive branching phenotype (3).

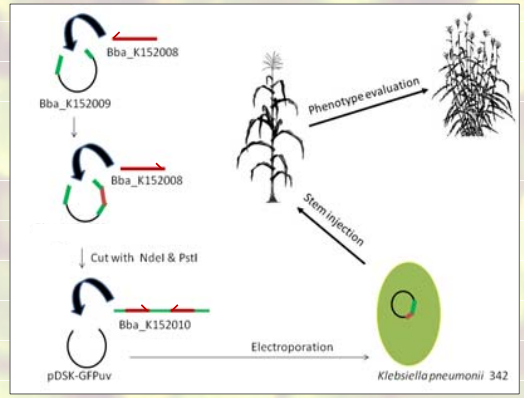

Figure 6: Methods used to build and test a BIGS construct against the corn TB1 gene.

Results: BIGS

To test our bacterial vector and delivery plasmid, we injected corn plants with pDSK-GFPuv tagged kp342 and evaluated plasmid maintenance and bacterial survival over a period of one month (figure 4). Based on colony forming unit counts (CFU) we injected 1.07 million microbes into the stem but observed two weeks later in leaf, crown root and seminal root about 12 microbes per milligram, while stem tissue contained 500 microbes per milligram. The bacteria appear to have been able to move systemically through their host, but not maintain the plasmid for the entire lifetime of the plant.

To build the BIGS construction tool, we designed an alternate restriction site pattern of NdeI-EcoRI-XbaI and NotI-SpeI-PstI around a central hairpin loop forming 100 bp *Zea mays* Actin1 intron, and included a transcriptional terminator based on BBa_B1006 (an error resulted in the pattern of SpeI-NotI-PstI instead). Production continued using template free PCR with 60 bp primer and 200 bp primers from IDT. The TB1 RNAi construct was made and electroporated into kp342 (figure 6). V2 stage corn were injected with 20 µl of this endophyte at OD600 = 0.8 and evaluated for phenotype two weeks later relative to tb1 mutants injected with pDKS-GFPuv tagged kp342 (figure 5).