PROGRESS REPORT

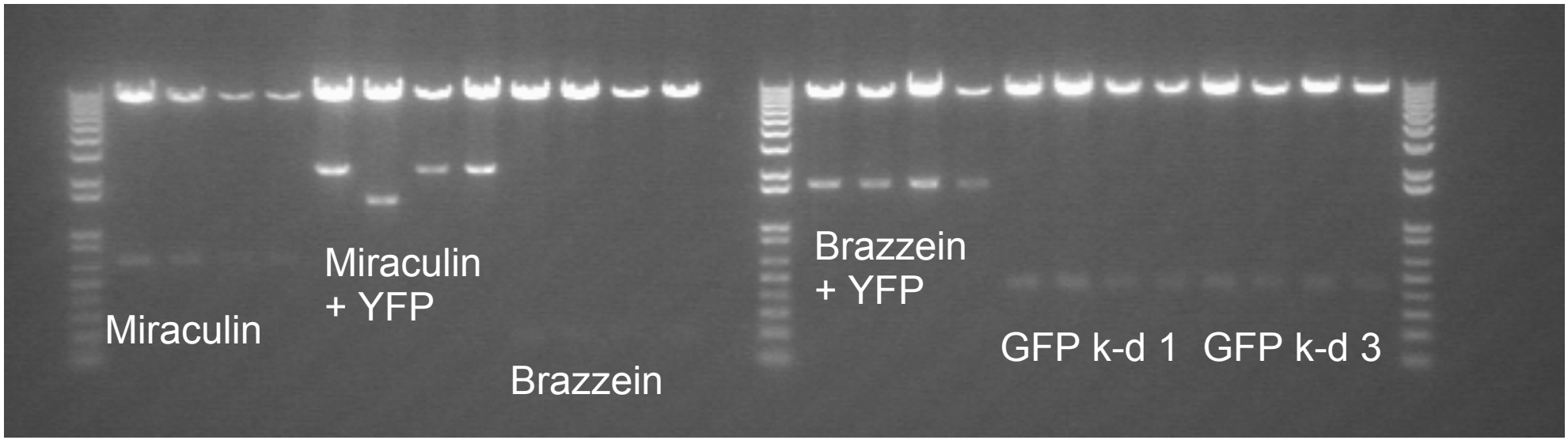
iGEM

July 19, 2010

Team Vector → Team Transform

Other teams have been finishing up constructs - we've taken six and transformed them into V9 and V10 (expression vectors)

- Miraculin (alone)
- Miraculin plus YFP tag
- Brazzein (alone)
- Brazzein plus YFP tag
- GFP Knockdown Construct 1
- GFP Knockdown Construct 3

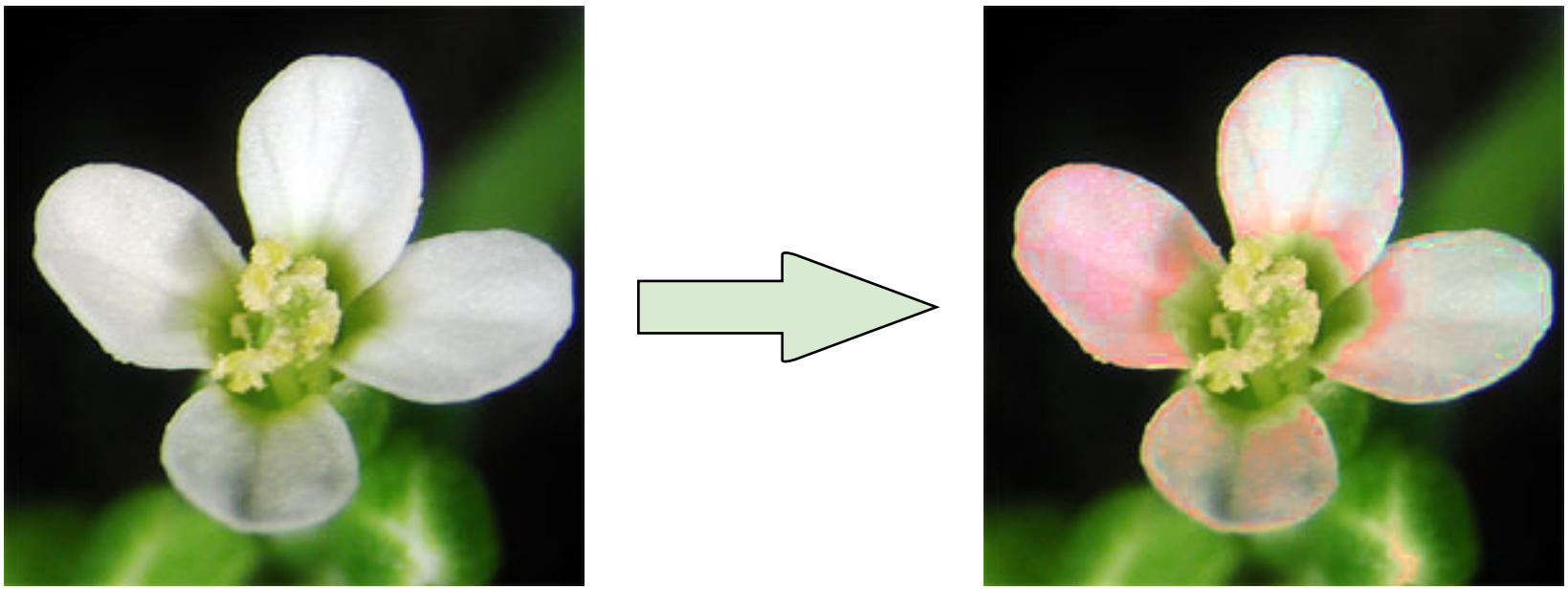


Digest Confirmation - all constucts except V23-2 appear correct

Team Vector → Team Color

New Project!

The Concept:

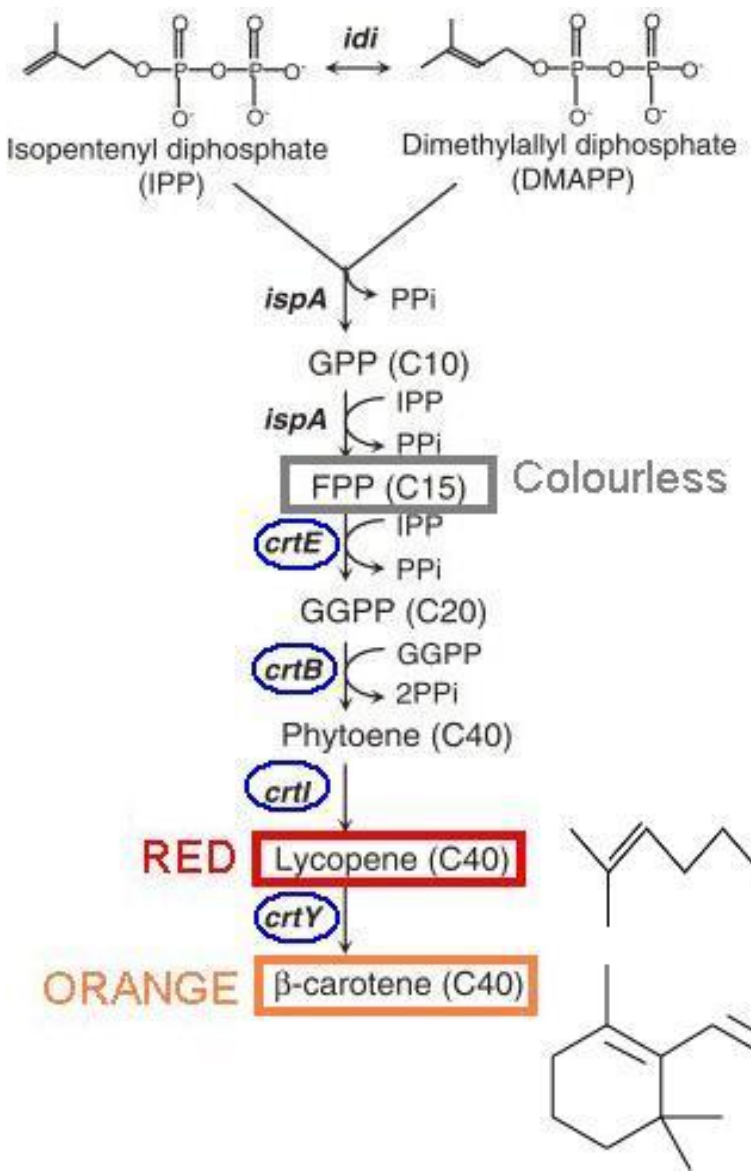


Altering the color of arabidopsis flowers through over-expression of pigments

Think orange flavored arabidopsis with orange flowers!

Team Color

The Plan:

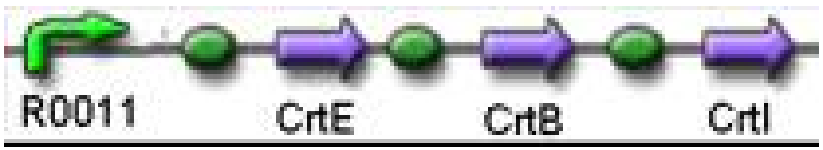


Stimulate the production of lycopene or beta-carotene by expressing key enzymes in the carotenoid metabolic pathway

Team Color

Ideas, Part I (Enzymes):

Cambridge 2009



Lycopene production

Beta-Carotene production

Problem - Bacterial Constructs...

translational and metabolic differences between prokaryotes and eukaryotes

Potential Solution - Reconstruct Device

reassemble putting promoters before each enzymatic step

Team Color

Ideas, Part I (Enzymes):

Situation - Carotenoid production already present in plants

- low levels in the flowers

Potential Solution - Over-express key enzymes in pathway

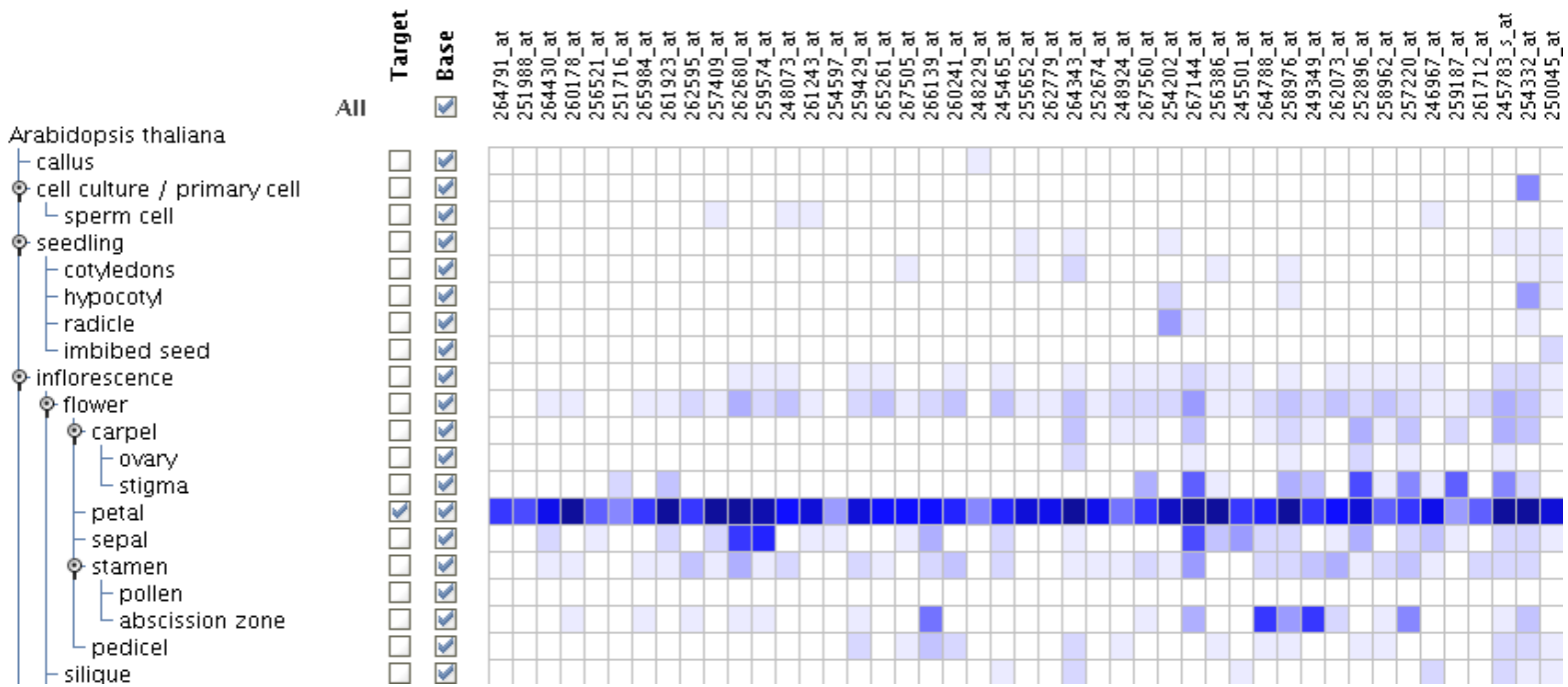
- use genevestigator to find weakly expressed parts of the pathway
- generate DNA sequences through PCR
- express individual enzymes *or* constructs of multiple enzymes in plants

Team Color

Ideas, Part II:

Promoters -

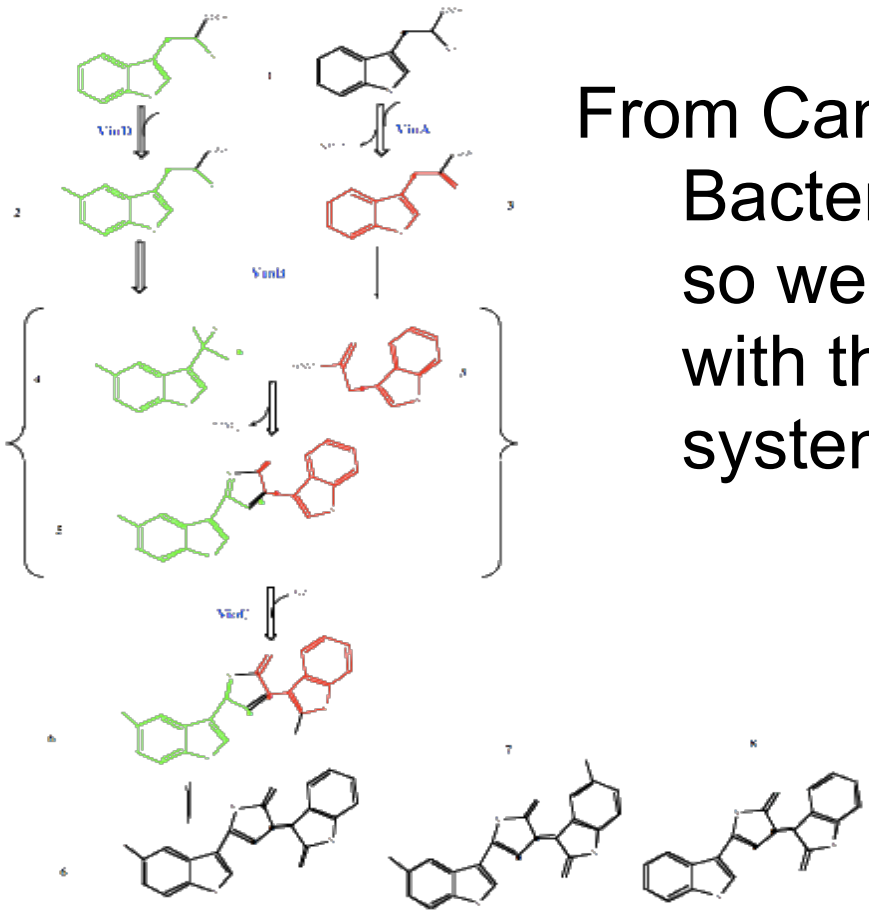
1. Use a non-specific, high-expression plant promoter, like with our other constructs
2. Find a flower specific promoter to localize expression to the petals.



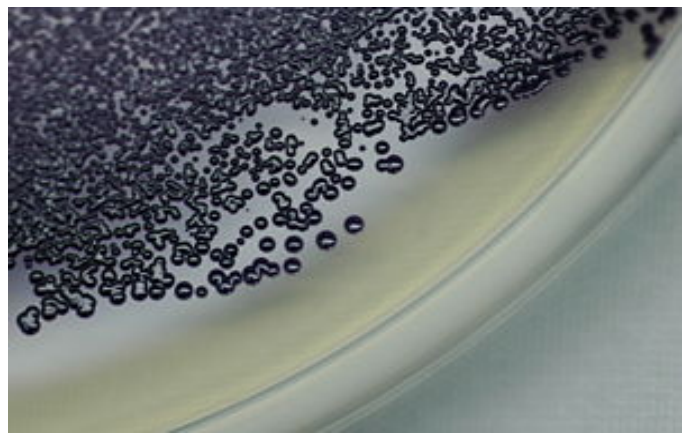
Team Color

Ideas, Part III:

Another Idea -



From Cambridge 2009 - Violacein Production Bacterial pathway with bacterial enzymes, so we may run into the same problems as with the existing carotenoid biobrick system.




LooM Flavor

Main Goals

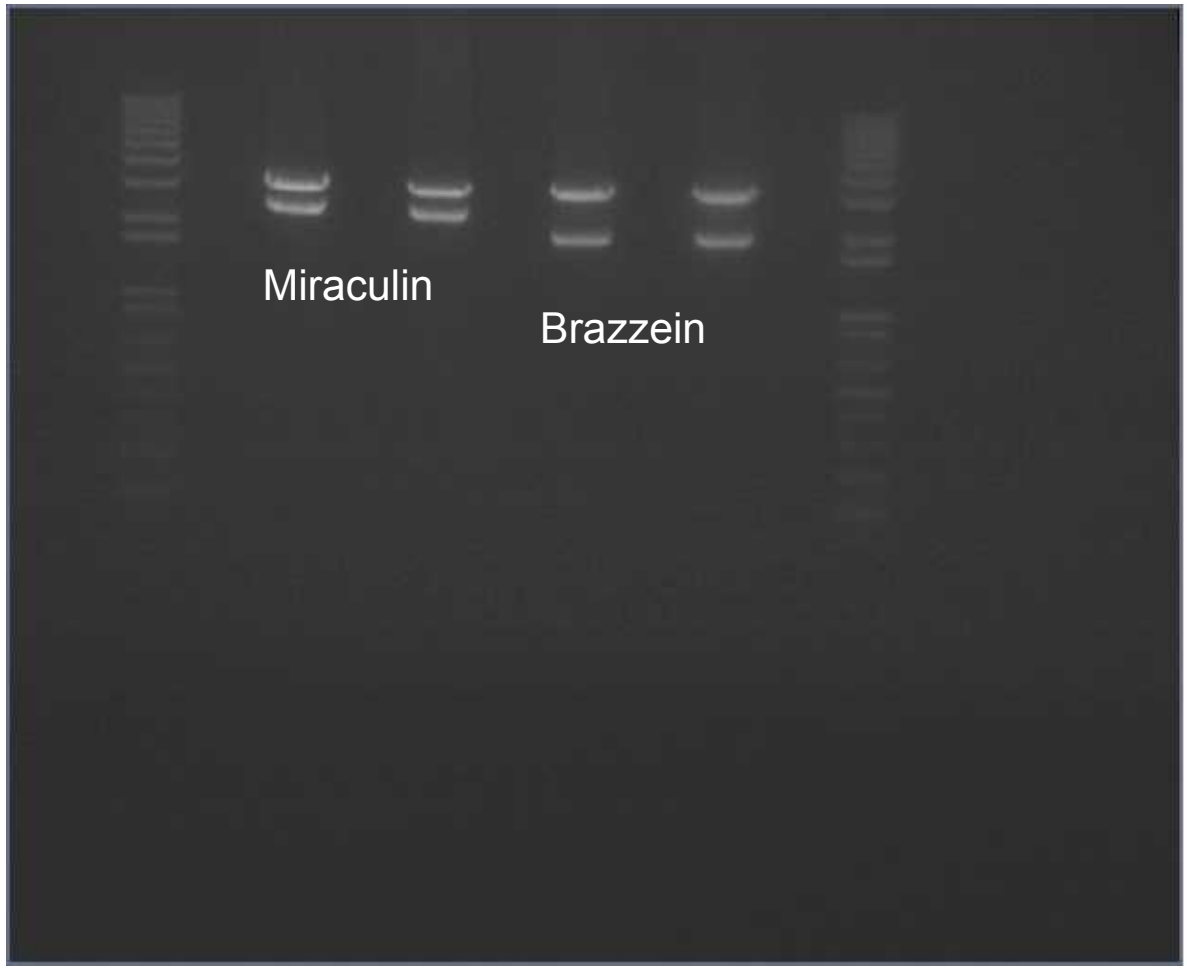
- Create BioBrick Parts: Ongoing
  - pORE vector parts being sequenced: Sequencing good
- Extract Valencene from Oranges: Genomic DNA extracted
- Express Miraculin, Brazzein: In Progress
  - Express in *E. Coli*: In Progress
    - Tag with *StrepII, YFP-2x*
  - Express in Plants: In Progress
- Express Wintergreen, Banana Scent Pathways: On Hold
  - Extract pieces of the pathways from BioBrick parts
  - Expressing in *E. Coli*, Plants

Last week

1. Miniprepped M/B+YFP+STOP+NOSt
2. Ligated M/B+YFP+STOP+NOSt into V24 (pETDUET)
3. Transformed and Miniprepped STOP biobrick part (from Karmella)
4. Ligated M/B+StrepII to the STOP bb part
5. Miniprepped (M/B+YFP+STOP+NOSt in V24)
6. Transformed (M/B+YFP+STOP+NOSt in V24) into lac inducible E. Coli
7. Extracted genomic DNA from valencia oranges (new kit)
8. PCR of valence - have not confirmed
9. Miniprep of M/B+StrepII+STOP - DID NOT WORK

Miraculin/Brazzein YFP and NosT & Stop

Ligation

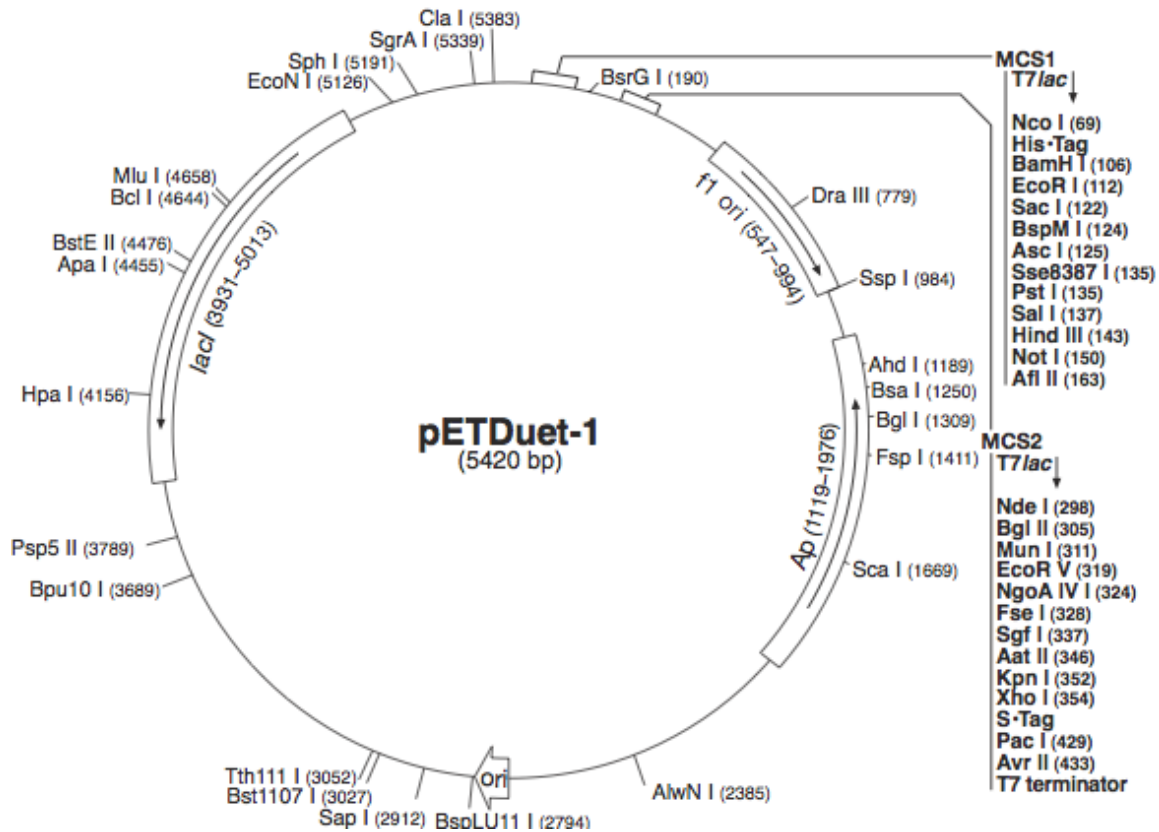


Confirmation digest looks good

Miraculin and Brazzein expression in e. coli

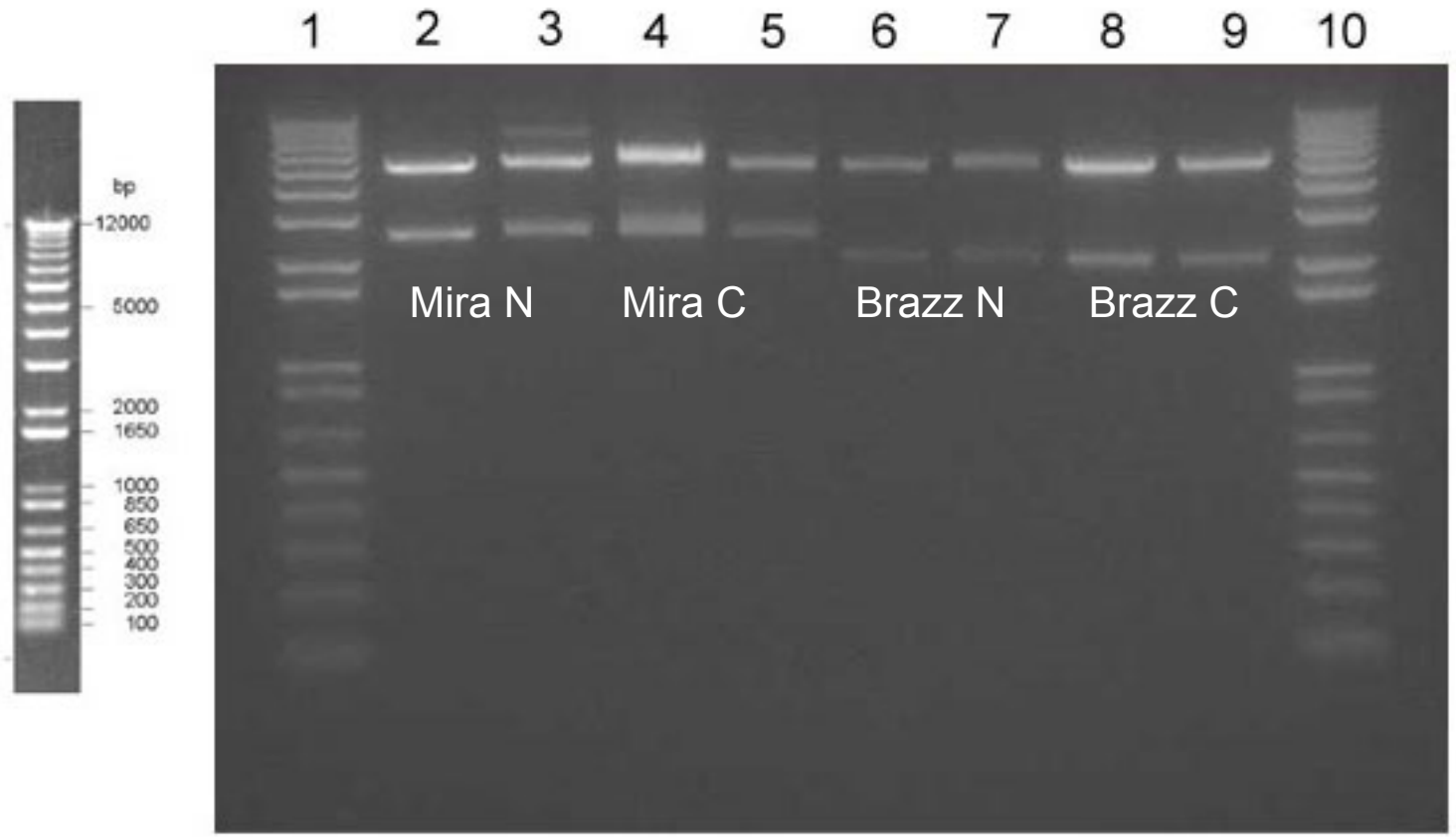
- Ligation of Mira/Brazz+YFP+NOSt & STOP construct into the V24 pETDUET vector

  - expression of proteins in IPTG inducible bacteria



Miraculin and Brazzein expression in e. coli

- Confirmation digestion gel looks good

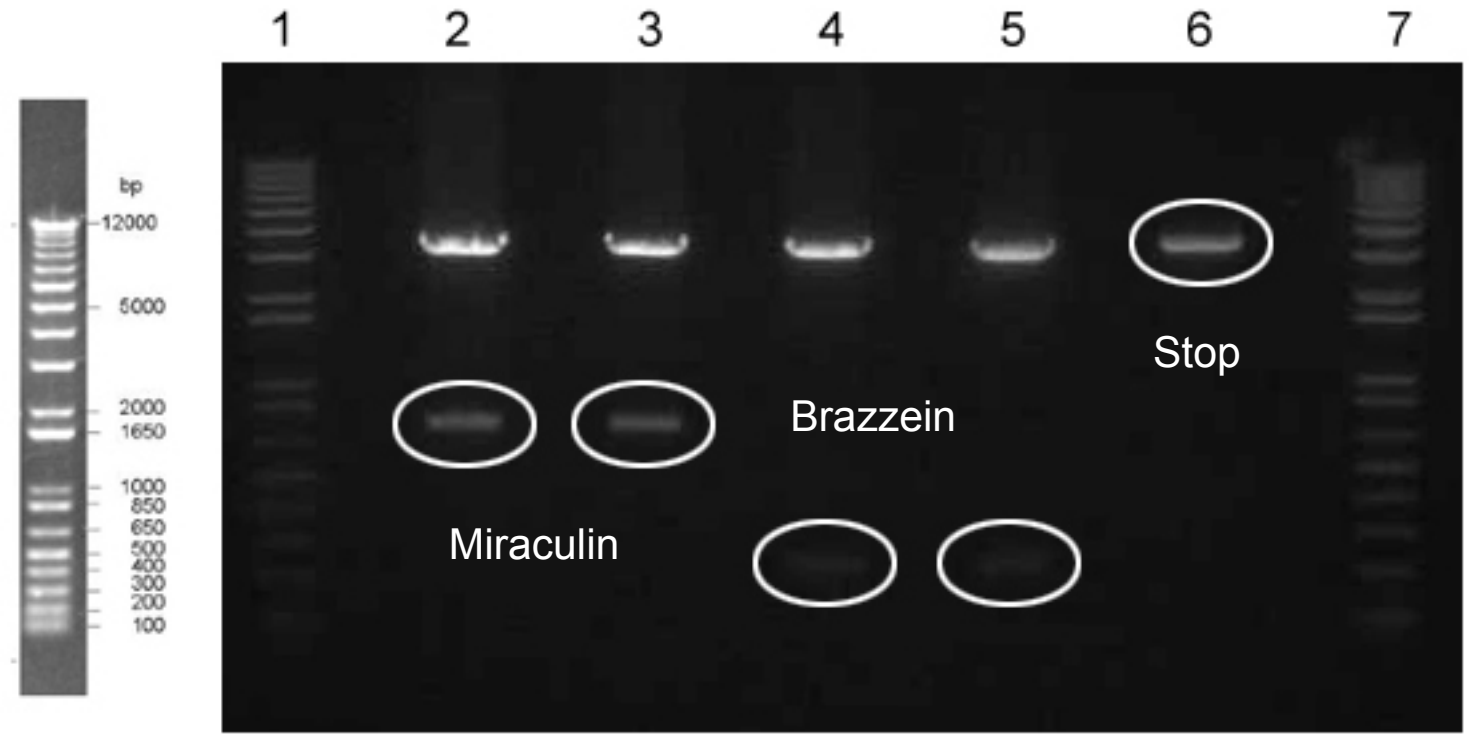


Expected Lengths
Miraculin + YFP + NOSt + STOP = 700 + 1500 + 250 = 2450 bp
Brazzein + YFP + NOSt + STOP = 300 + 1500 + 250 = 2050 bp
V24 = 5200 bp

Expression in lac inducible *E. Coli*

- *E.Coli* is ready to be induced and observed (will do today and tomorrow)
- No YFP was seen in colonies (without IPTG induction), but this does not necessarily mean failed expression

Addition of stop codon to Miraculin and Brazzein + StrepII constructs

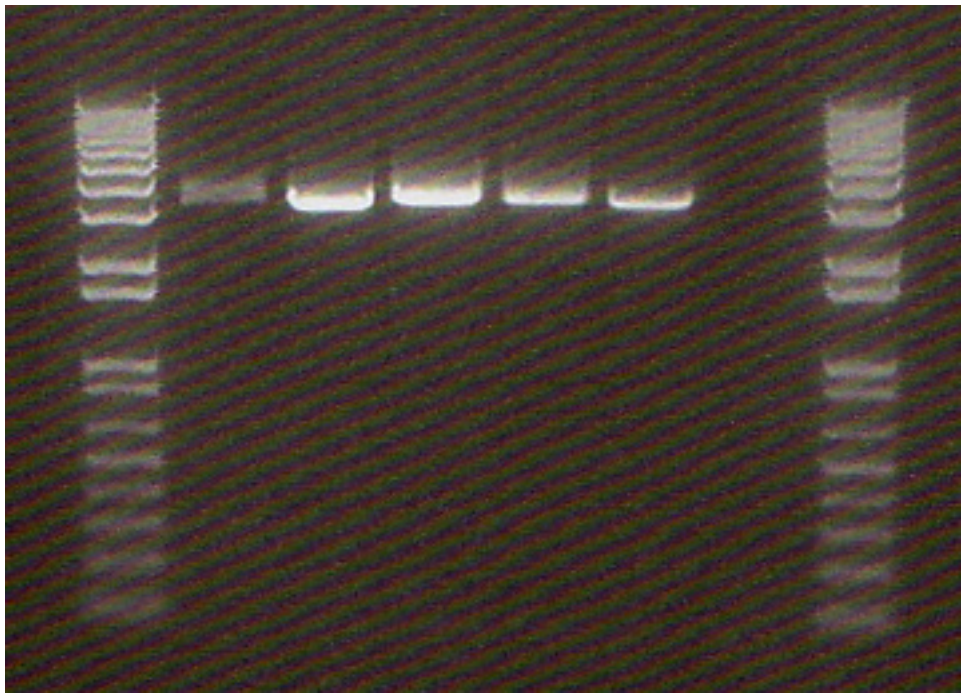


- Used stop as vector and Miraculin/Brazzein as insert

Miraculin/Brazzein + StrepII + STOP

Confirmation Digest with Xba1/Pst1

1. Ladder
2. BN1
3. BN2
4. BC1
5. BC2
6. MC2
7. blank
8. Ladder



- Should have seen inserts of 'Protein + StrepII tag' = ~750 (Miraculin) and ~250 (Brazzein)

Conclusion: Miraculin Brazzein insert did not ligate properly.

To Do This Week:

1. Grow cultures of pDUET *E.Coli* to induce with IPTG, visualize
2. Re-ligate Mira/Brazz+StrepII into V0120 backbone
    1. Insert Mira/Brazz+StrepII+STOP into pDUET vector, express in bacteria
3. Confirm PCR of Valencene from extracted genomic DNA
    1. If good: insert as BioBrick part, sequence to confirm
    2. If bad: re-do DNA extraction (made mistakes with kit last time)

Progress/Future Directions

Identify Allergens

Design Primers for shRNA

PCR out our intron, antisense, sense equences

amiRNA

Clone into Biobrick vector (VO210)

Transform into E. coli

Insert into agrobacterium

Sequence

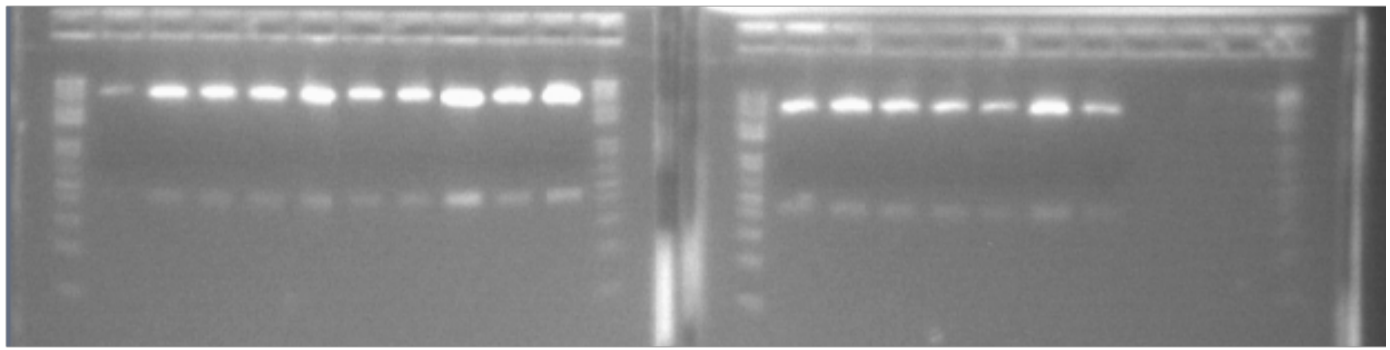
Transform into Arabidopsis/ Respective Plants

Observe Allergen Knockdown (quantitatve pcr)

Progress

- amiRNA
  - Gave amiRNA (GFP) to insert into agrobacterium vector
  - Have a new set to send out for sequencing
- ihpRNA
  - Genomic DNA extraction (Pme+ PaI as well)
  - Ready to send out all sense/antisense parts for sequencing
  - Added LTPS+ PDK (need to check on sequencing)

Genomic DNA extraction



We picked more colonies of the amiRNA parts


Team Fence

Design Genetic Fence

Design and order primers to amplify parts + have other parts synthesized

PCR and digest then ligate and clone in the parts

Clone into Biobrick vector (VO210)

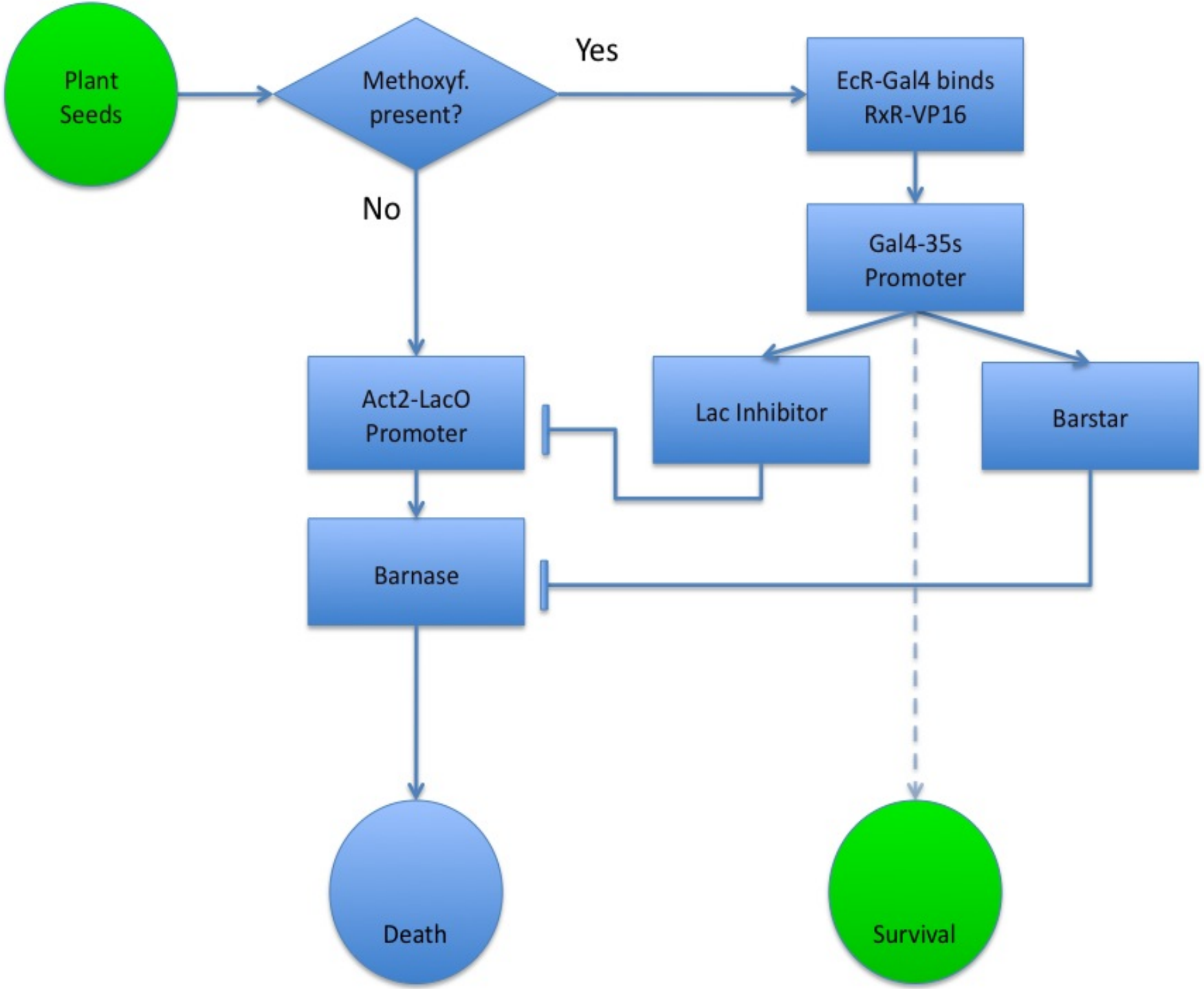
Transform subcomponents into yeast

Transform into E.coli

Transform into agro-bacterium

Transform into Arabidopsis/ Plants

Test fence elements individually

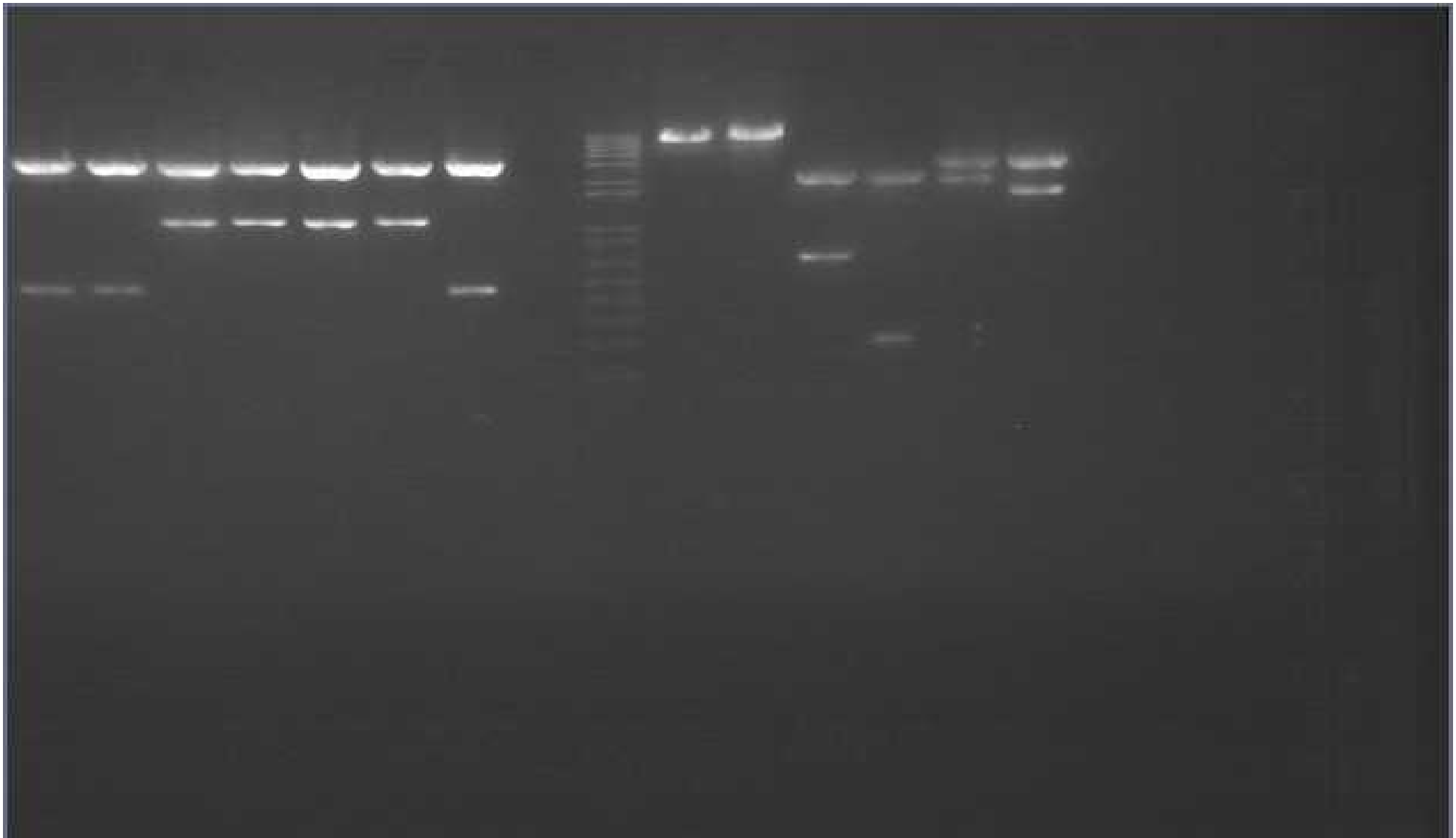

Plant Seeds
Methoxyf. present?
Yes
No
EcR-Gal4 binds RxR-VP16
Gal4-35s Promoter
Act2-LacO Promoter
Lac Inhibitor
Barstar
Barnase
Death
Survival

Progress

- Last week's ligations successful, sent for sequencing
- Ordered primers to biobrick and finish parts ordered for synthesis (RXRhm, RXRlc, EcR)
- Re-doing NLS ligations with new primers --> in progress
- Arabidopsis promoter PCR
- Degradation Signal
- Yeast Backbone Extraction

Ligations from last week

- Looked good, sent Barnase, Gal4 and LacIN for sequencing
  - Problems with LacIN

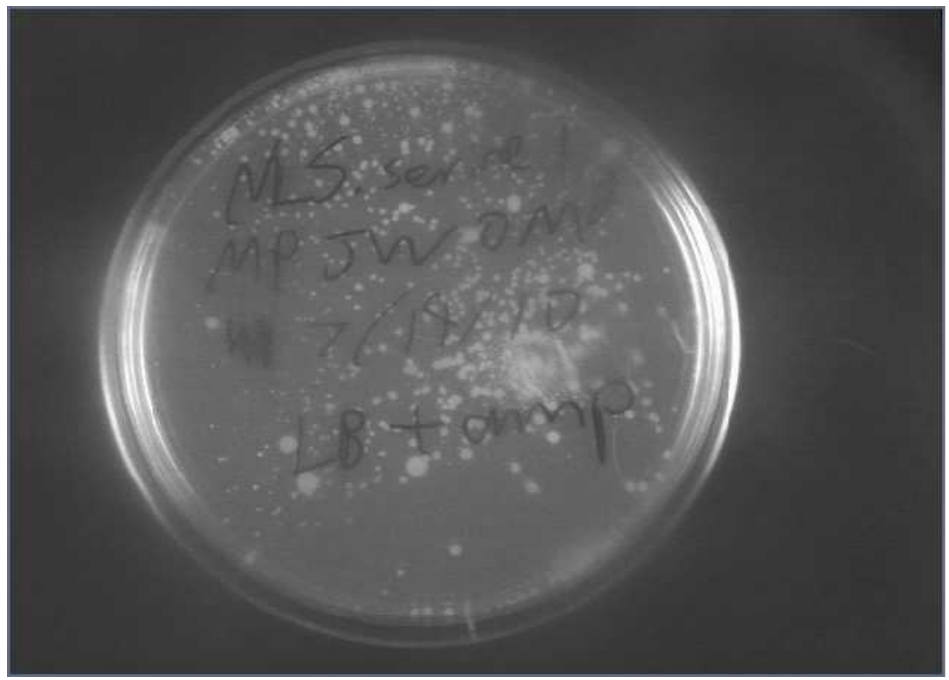


NLS biobricking, take 4

- After several previous attempts failed, new primers ordered last week
- Annealed new NLS primers
  - These primers removed a methylation site present in the previous primers
- Ligated with a 2 to 1 insert-backbone ratio, transformed

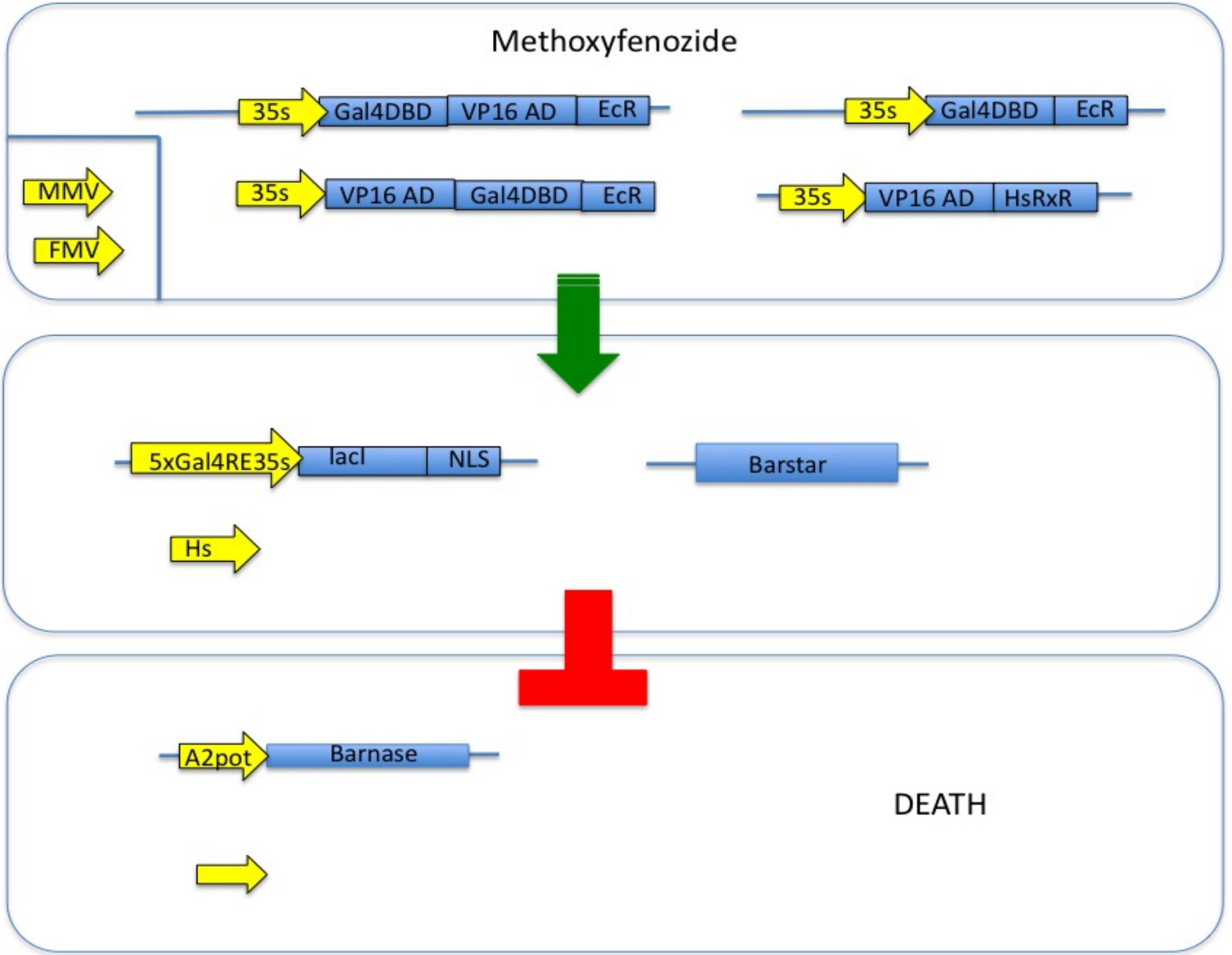
NLS biobricking, take 4

- Lots of non-specific colonies
  - recently poured LB + Amp plates?
  - Contaimination?
  - Something else?
- Further attempts on hold, as biobricking is not strictly neccessary for our fence

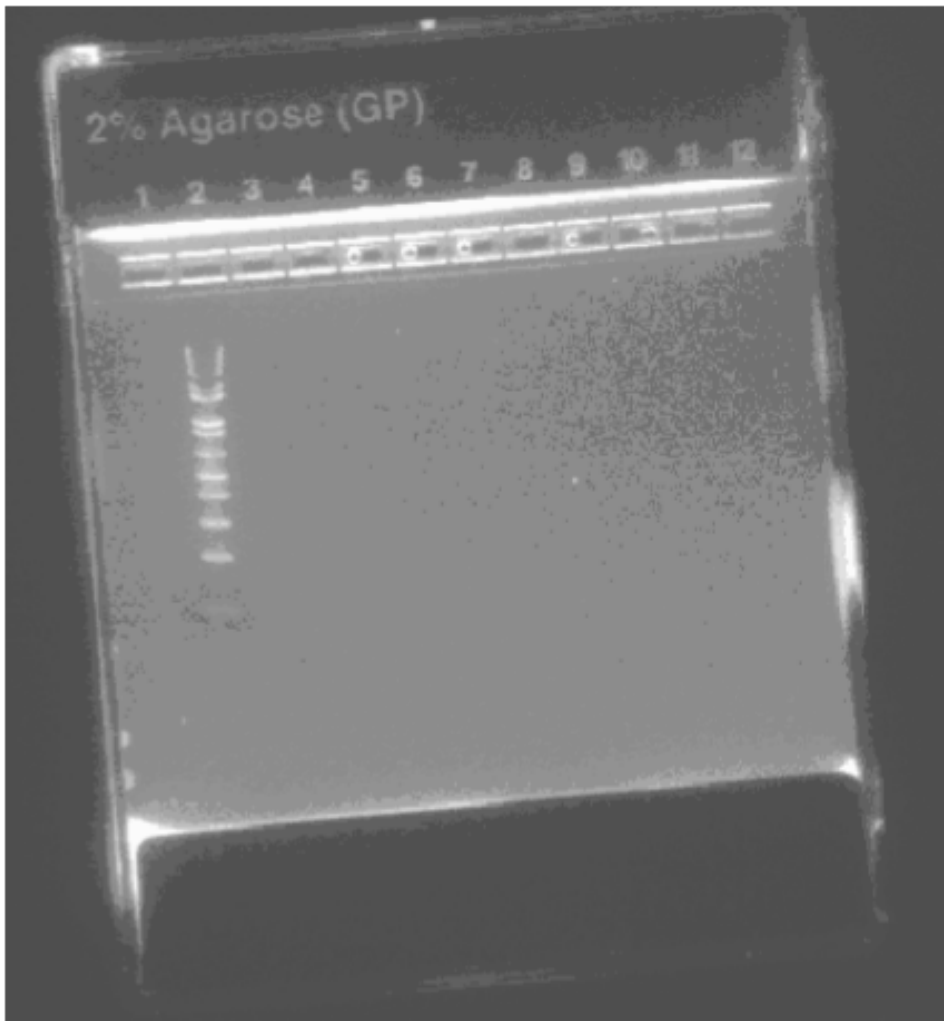


Preparing for Synthesis products

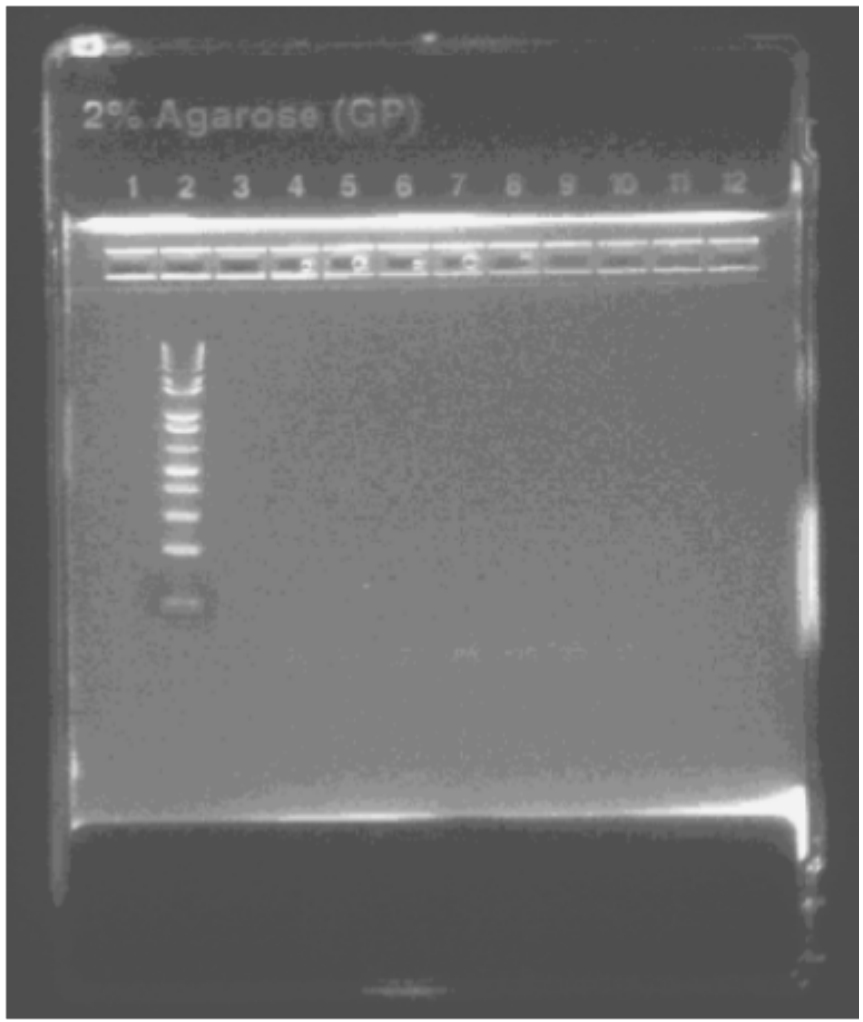
- Designed and ordered primers to finalize parts ordered for synthesis
  - adding start/stop codons, cut sites to prepare:
- VP16 - RXRhm and RXRlc
- Gal4 - EcR
- Annealed Gal4 promoter oligos


Methoxyfenozide
35s
Gal4DBD
VP16 AD
EcR
35s
Gal4DBD
EcR
MMV
35s
VP16 AD
Gal4DBD
EcR
35s
VP16 AD
HsRxR
FMV
5xGal4RE35s
lacI
NLS
Barstar
Hs
A2pot
Barnase
DEATH

PCR: 7/14 no product

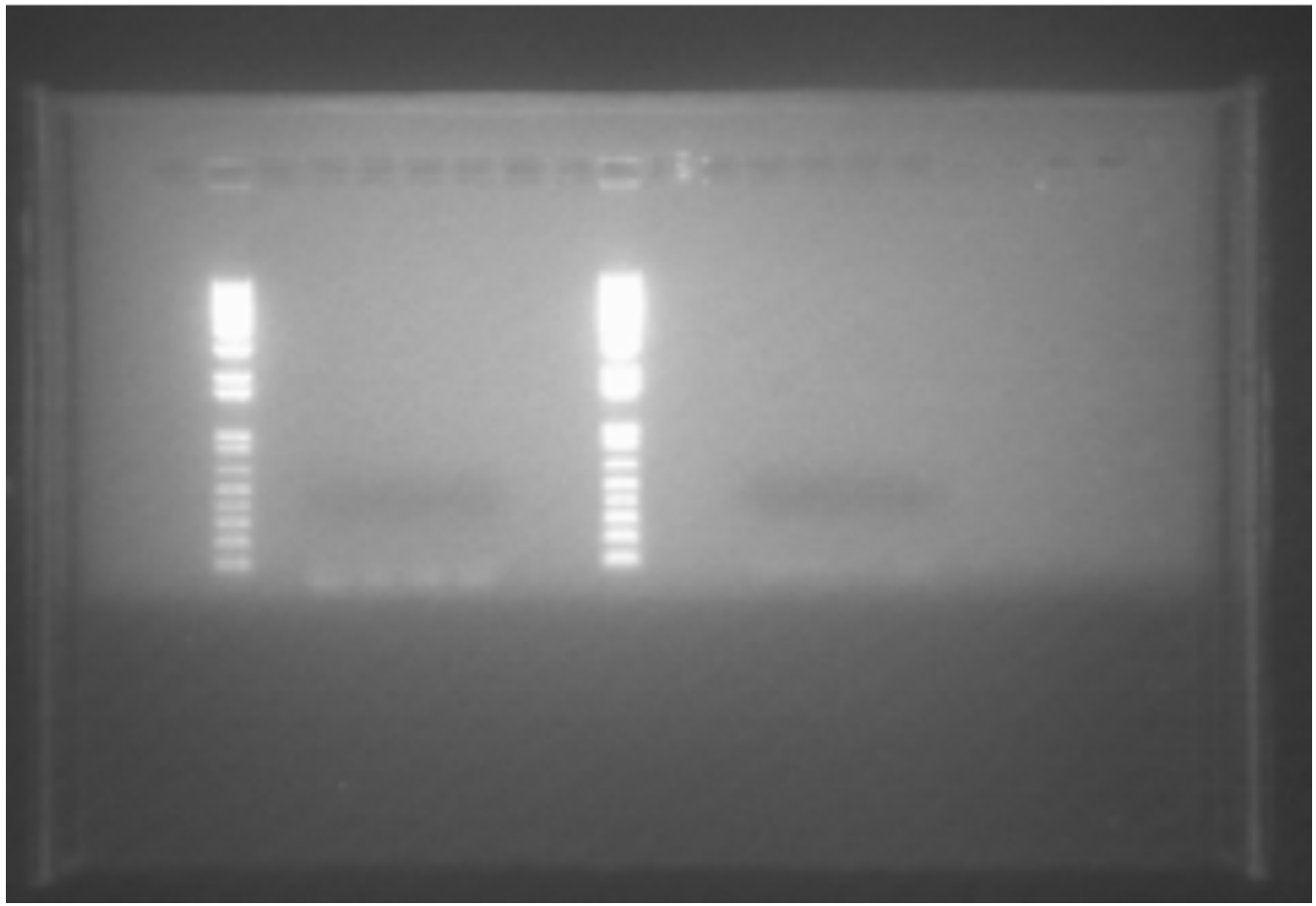


AtExp
2



AtArp
2

7/16: second attempt, more DNA

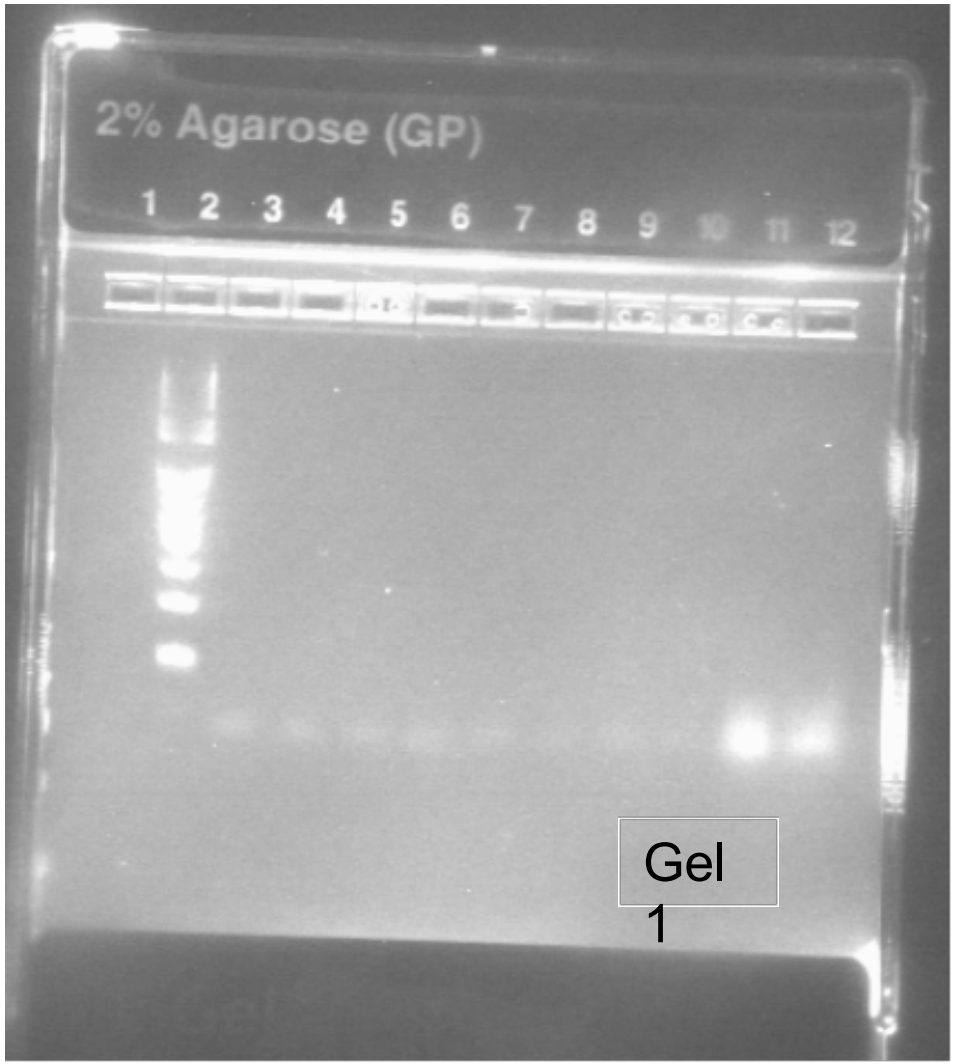


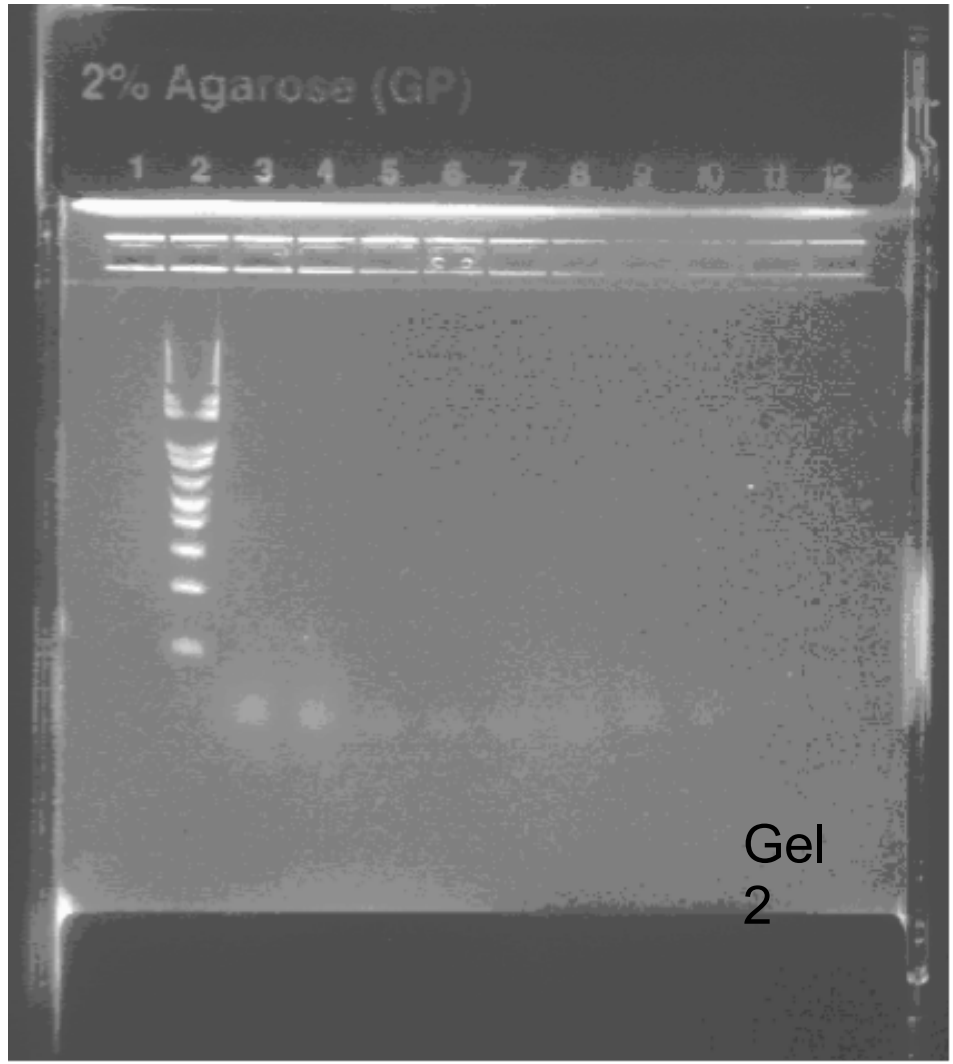
FAIL

DNA Extraction

- Zymo Research Plant/Seed DNA Mini Kit
  - Nanodrop values:
    - ~20 ng/microliter
    - 260/280: 0.9 to 1.1
- Phenol Chloroform DNA Extraction
  - No DNA
- QIAgen DNeasy Plant Minikit (liquid nitrogen)
  - Nanodrop values:
    - ~6 ng/microliter
    - 260/280: 1.42 to 1.48

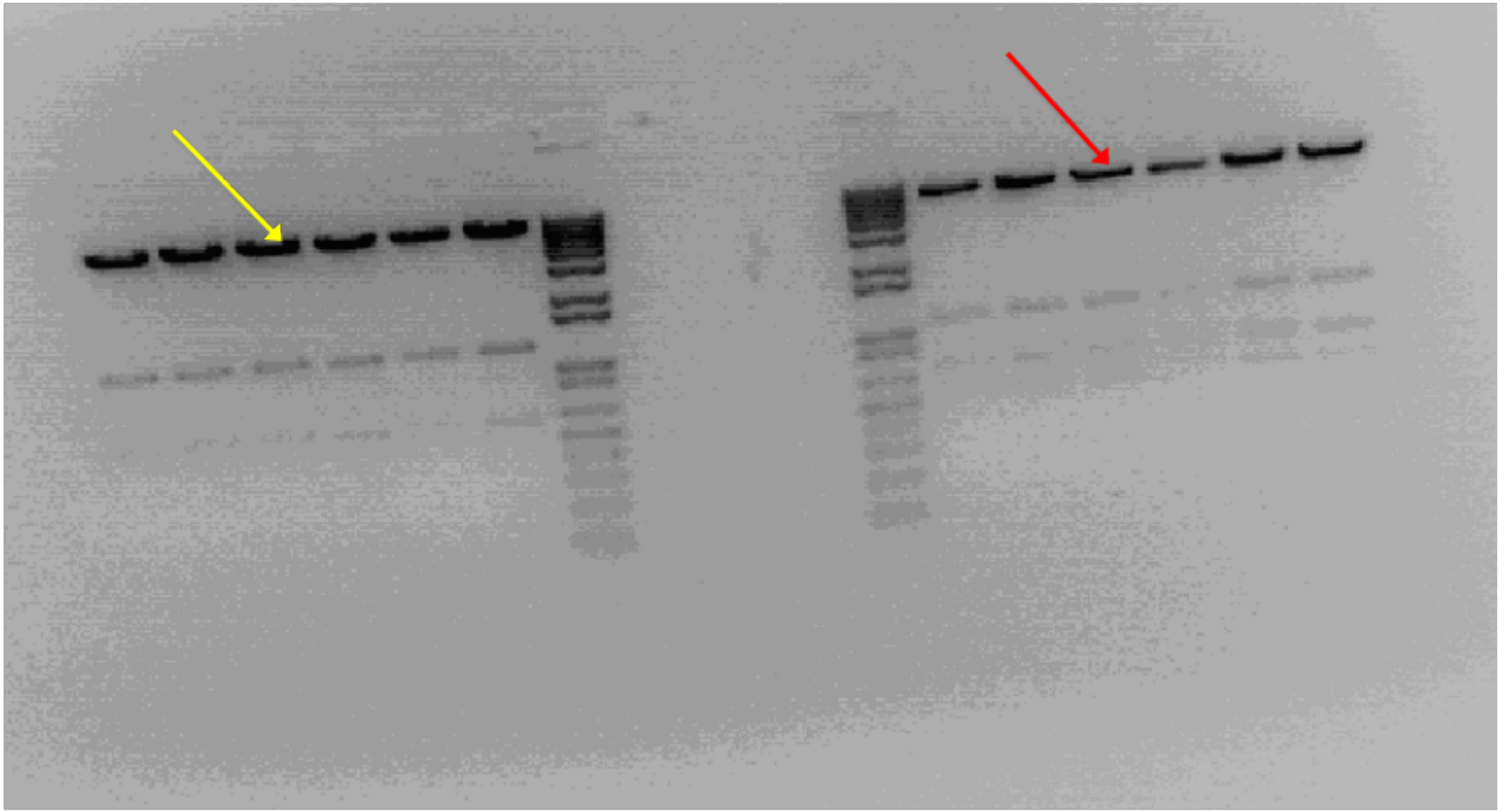
PCR: newly extracted DNA





lanes 11 & 12 of Gel 1 + lanes 3 & 4 of gel 2 contain control samples from team allergy

Yeast Backbone



Successfully isolated and extracted!

FEBS Letters 581 (2007) 3189–3196

PRT6/At5g02310 encodes an *Arabidopsis* ubiquitin ligase of the N-end rule pathway with arginine specificity and is not the *CER3* locus

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Abstract **The eukaryotic N-end rule pathway mediates ubiquitin- and proteasome-dependent turnover of proteins with a bulky amino-terminal residue. *Arabidopsis* locus At5g02310 shows significant similarity to the yeast N-end rule ligase Ubr1. We demonstrate that At5g02310 is a ubiquitin ligase and mediates degradation of proteins with amino-terminal Arg residue. Unlike Ubr1, the *Arabidopsis* protein does not participate in degradation of proteins with amino-terminal Phe or Leu. This modified target specificity coincides with characteristic differences in domain structure. In contrast to previous publications, our data indicate that At5g02310 is not identical to *CER3*, a gene involved in establishment of a protective surface wax layer. At5g02310 has therefore been re-designated *PROTEOLYSIS 6* (*PRT6*), in accordance with its ubiquitin ligase function.**
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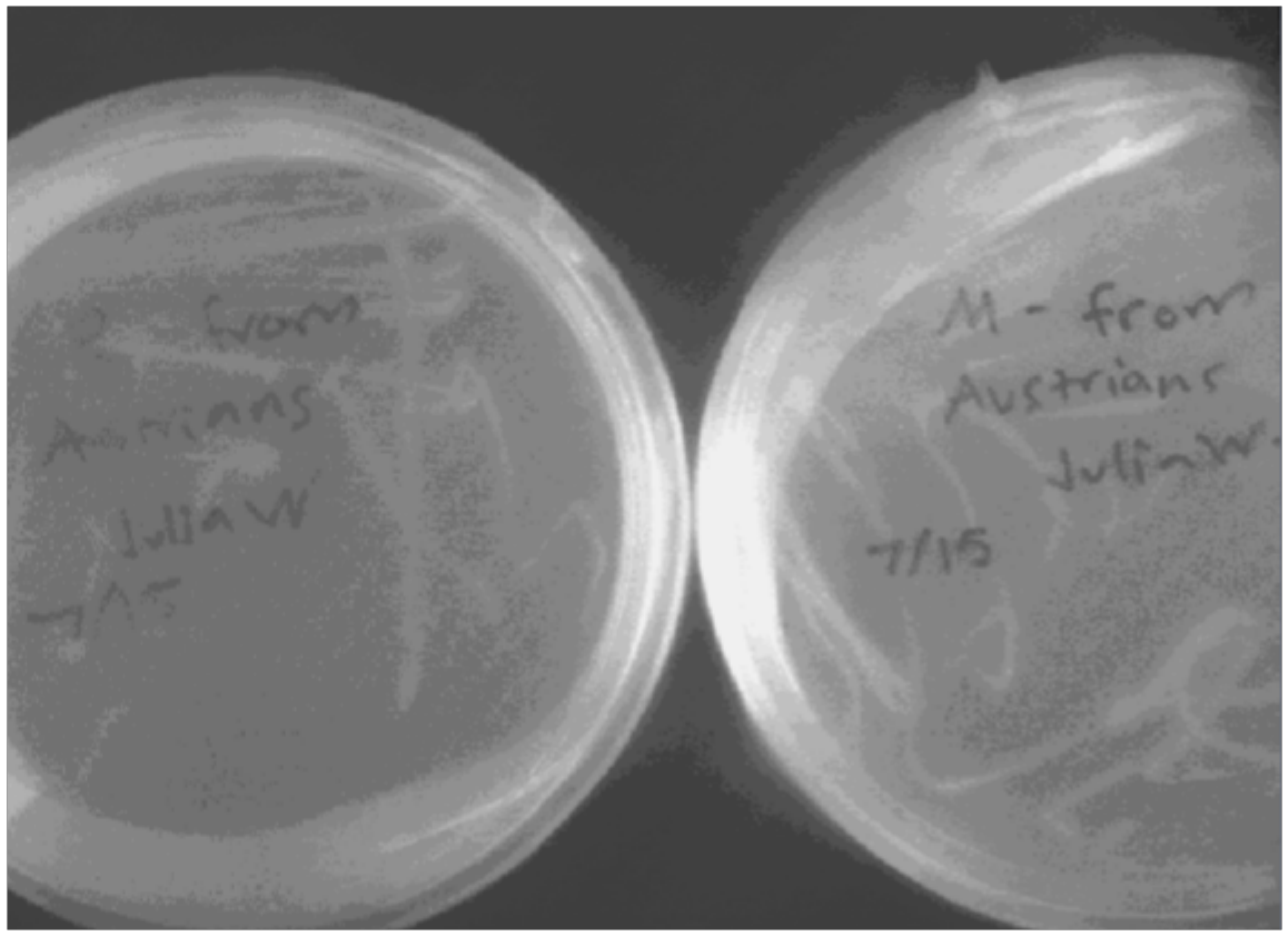
Keywords: *CER3*; N-end rule; Ubiquitin; Wax biosynthesis; *Arabidopsis*

Degradation Signal

- Colonies
- Innoculation
- Miniprep
  - 212 to 440 ng/ul



NEW IDEA: POSITIVE REGULATION OF TRANSGENE EXPRESSION IN YEAST