iGEM 2010 Progress Report

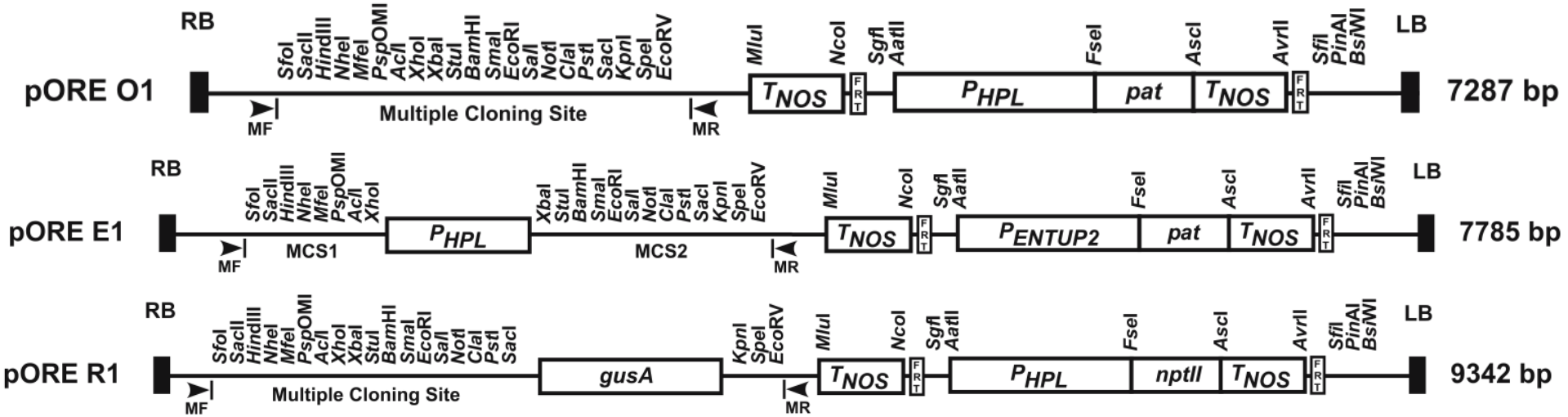
June 21, 2010

HARVARD iGEM

TEAM VECTOR

Aim: biobrick agrobacterium vector

We are working with 6 different plasmids known as pORE:



To achieve our aim we need to remove the MCS and replace it with the biobrick cloning sites

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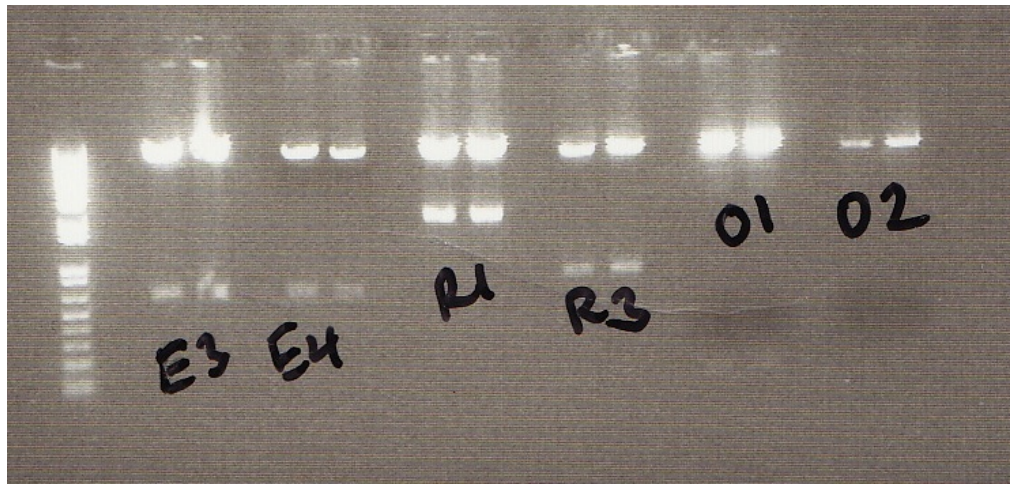
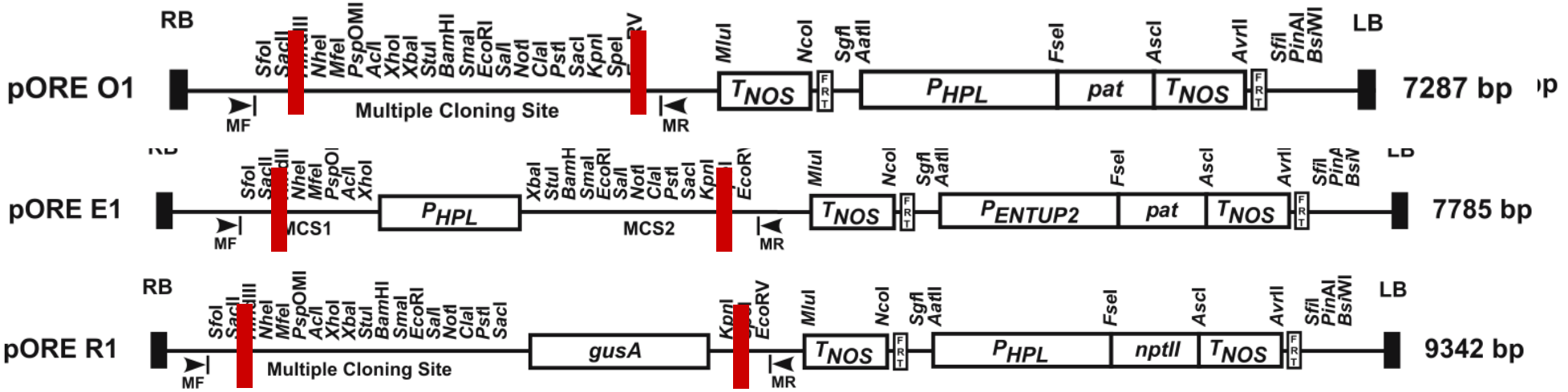
Progress so far: Backbones

Used restriction digests to remove MCS + promoters/ reporters from each plasmid, leaving the backbone.

- Open Series: cut with SacII and SpeI
- Reporter Series: cut with HindIII and SpeI
- Expression Series: cut with HindIII and SpeI





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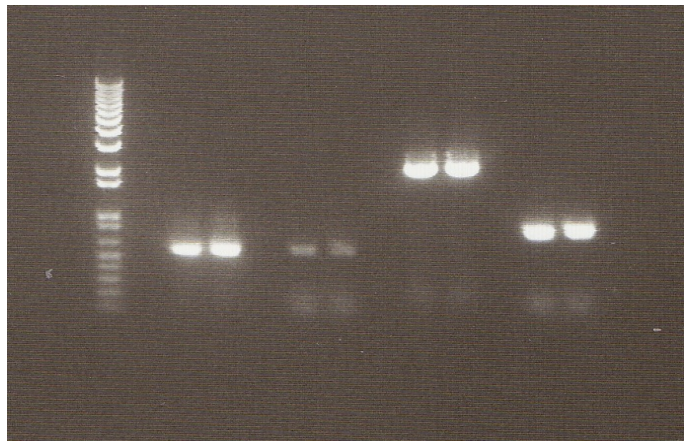
Progress: Inserts for reporter and expression series
Created inserts from primers via PCR on the original agrobacterium vector designed to retain any reporter or promoter already in the vector and add the Biobrick Multiple Cloning Site



E3 E4 R1 R3

- Reporter Series:
  - forward: HindIII cut site, Biobrick MCS, first part of appropriate reporter
  - reverse: last part of reporter, Nhe cut site
- Expression Series:
  - forward: HindIII cut site, first part of promoter
  - reverse: last part of promoter, Biobrick MCS, Nhe cut site

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Progress so far: Ligation of inserts and backbones

- Ligated inserts and backbones of expression and reporter series vectors to create biobrick agrobacterium plasmids.
- Transformed into NEB Turbo Competent E. coli cells.
- Picked colonies and made cultures.

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Next steps:

- Create inserts for O1 and O2 by annealing oligos and digest with SacII and NheI.
- Ligate O1 and O2 backbones and inserts.
- Transform resulting O1 and O2 plasmids into E. coli.
- Transform each plasmid into agrobacteria.
- Sequence constructs to check for accuracy

Next: Team Flavor

TEAM FLAVOR

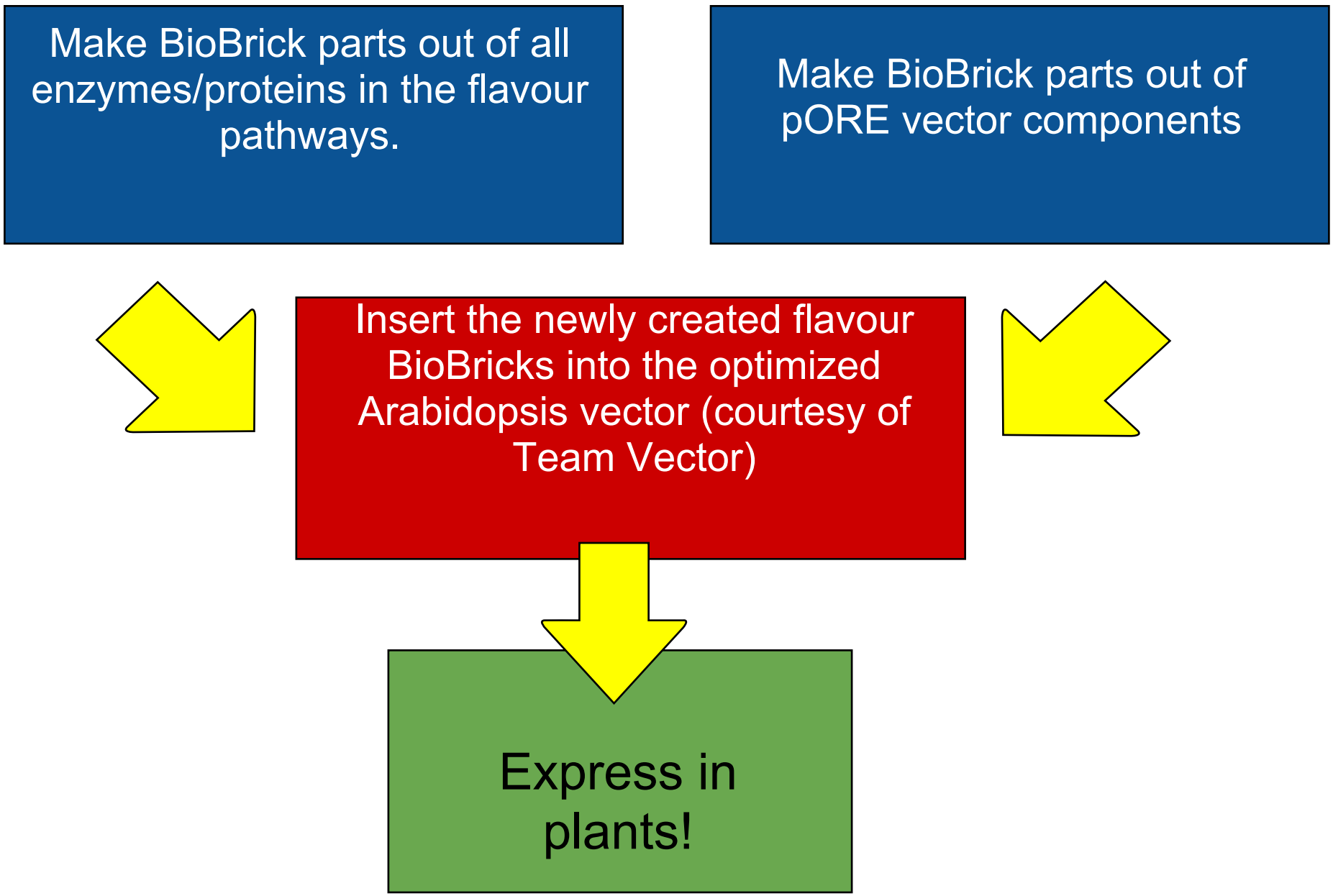
Our Plan:

- Express different flavors in plants
  - Targeting and altering specific metabolic pathways
  - Producing proteins
- Make BioBrick Parts

Flavors:

- Miraculin
  - Turns 'Sour' to 'Sweet'
  - Ordered from Mr. Gene
  - Arrival Pending... (should be early this week)
- Brazzein
  - 500x to 2000x sweeter than sugar (Wikipedia)
  - Ordered from Mr. Gene
  - Arrival Pending... (should be early this week)
- Valencene
  - Orange/Citrus smell, derived from Valencia oranges
  - Extracted from oranges ourselves
- Wintergreen Scent
  - From the 2006 MIT iGEM team
  - Full pathway available in 2010 iGEM kit
- Banana Scent
  - From the 2006 MIT iGEM team
  - Parts of pathway available in 2010 kit

To Do:


Make BioBrick parts out of all enzymes/proteins in the flavour pathways.
Make BioBrick parts out of pORE vector components
Insert the newly created flavour BioBricks into the optimized Arabidopsis vector (courtesy of Team Vector)
Express in plants!

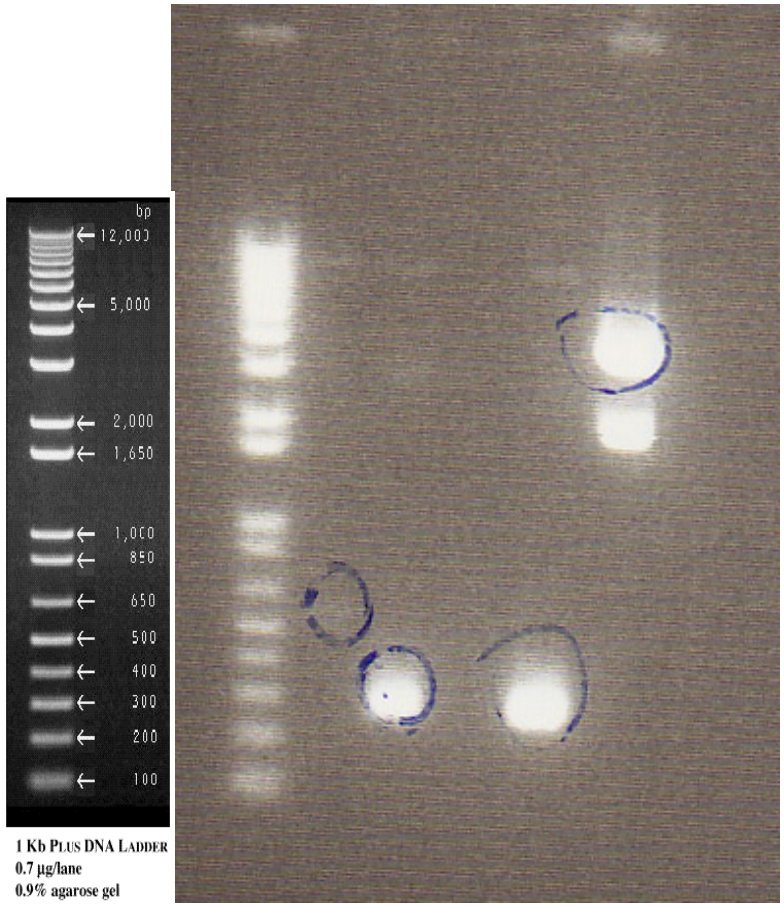
What we have done this week:

1. BioBrick parts from the registry were transformed and expressed in E. Coli:
    - Full and partial wintergreen pathway
        - Very few colonies
    - Parts of banana pathway
        - Expressed components of banana pathway did not grow!
        - PCR of restriction digest yielded products of incorrect size
2. Primers were designed, and PCR preformed on components of the e3 pORE vector
    - Promoter
    - Terminator sequence
    - Terminator sequence + Stop codon
        - We are in the process of inserting these components into standard BioBrick vectors (V0120). First attempt at ligating into vector did not work. Result of second ligation attempt will be seen on Monday.

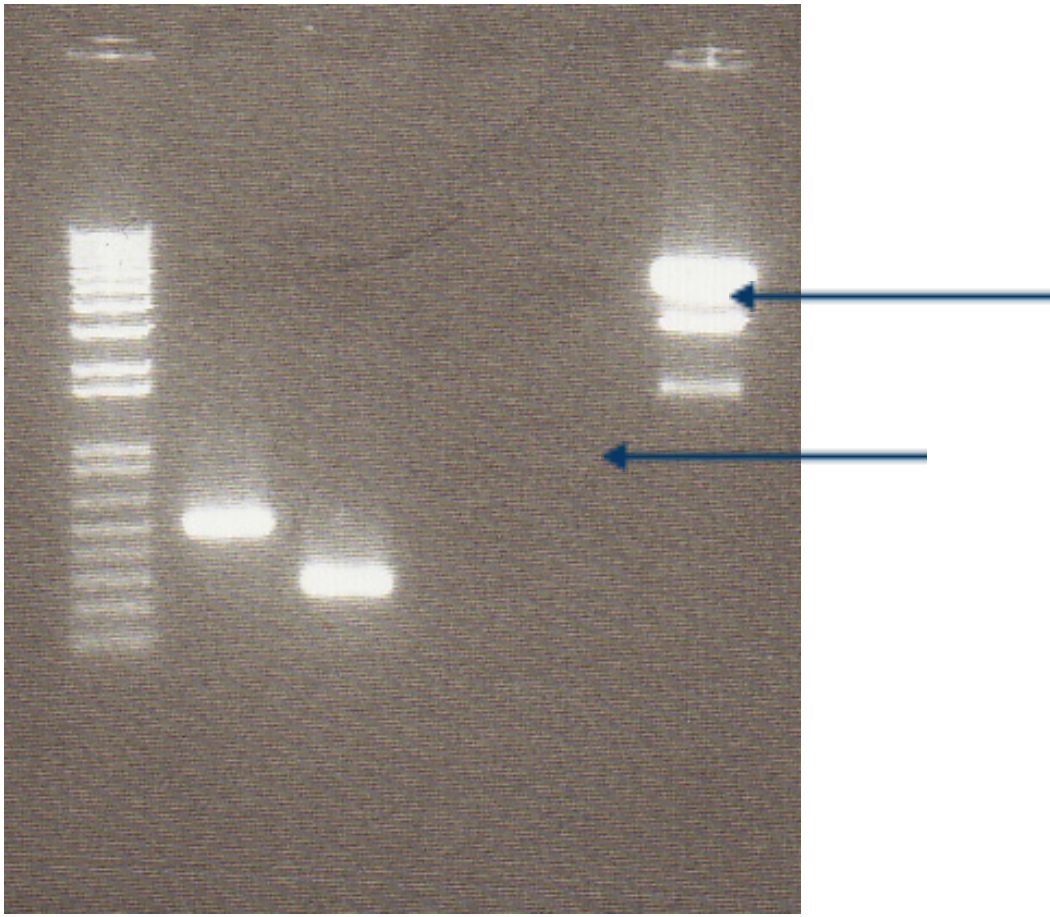
Digestion of PCR Products

1. Ladder
2. Promoter pENTCUP2
3. NOSt
4. empty
5. NOSt + STOP
6. Plasmid

Ladder 1 2 3 4 5 6

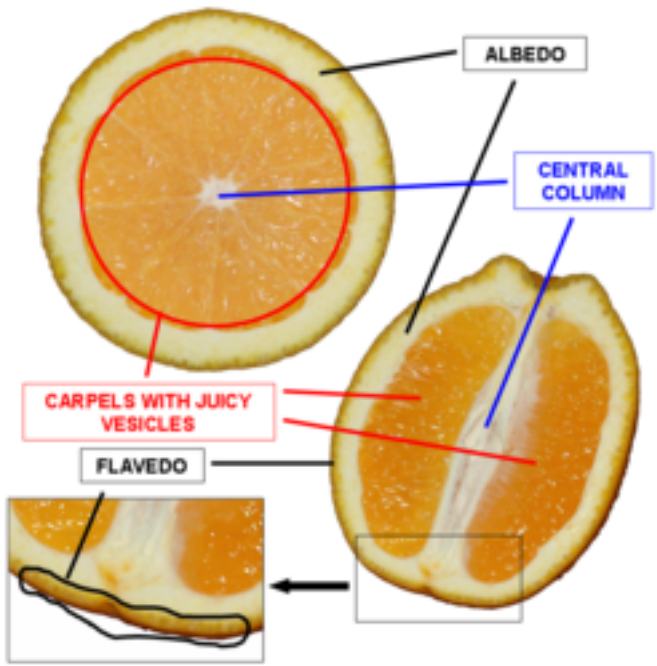


1 2 3 4 5 6



What we have done this week (cont'd):

3. Valencia orange RNA extraction and cDNA synthesis
    - extracted RNA from flavedo of Valencia orange
    - need to remove pstI sites from valencene gene without altering functionality
        - site directed mutagenesis
        - primers have been ordered



Primers for site directed mutagenesis:

Valencene synthase t30a (amino acid 440 Ile -> Ile)
5' gagaaacatttcgtcctacagcagatttccatcctagtt 3'

Valencene synthase c1320a (amino acid 10 Thr -> Thr)
5' aggttgtaaaagcagcatcagttatatgcagactcatgga 3'

Team Allergy

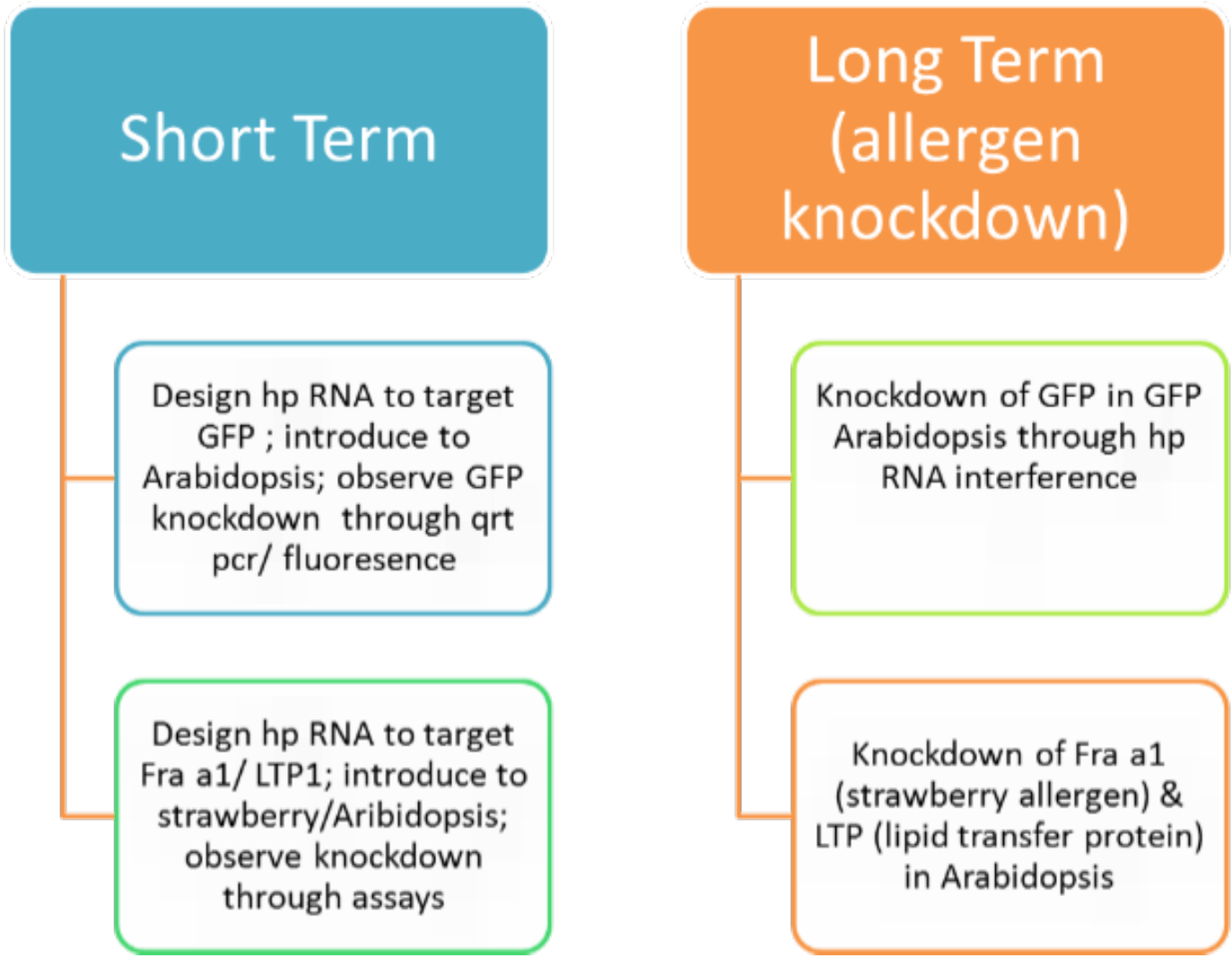
Identify Allergens → Design Primers for shRNA → PCR out our intron, antisense, sense sequences

PCR out our intron, antisense, sense sequences → Clone into Biobrick vector (VO210)

Clone into Biobrick vector (VO210) → Transform into E. coli → Transform into agrobacterium

Transform into agrobacterium → Transform into Arabidopsis/ Plants → Observe Allergen Knockdown

Team Allergy Goals



What We Did...

Monday and Tuesday :

RNA extraction on strawberry fruit obtained from Shaws
Obtained sufficient mRNA to yield the following amounts of cDNA

Sample #	cDNA concentration (ng/uL)	mRNA concentration (ng/uL)	Part of the strawberry
1	441	11	Outer Receptacle
2	1477	36	Receptacle
3	2749	6.5	Inner Receptacle

PCR

Wednesday and Thursday:

Tried to PCR out Fraa 1 for biobrick cloning using cDNA from strawberries

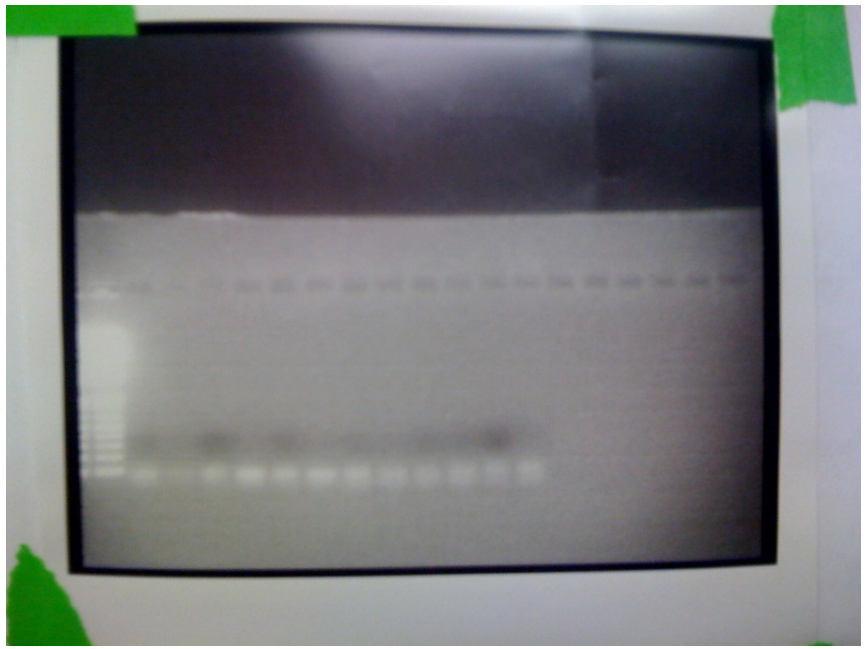
Goal: 300bp DNA sequence (Fraa1)

Result?

Unsuccessful --- Primer Dimers? PCR products were ~ 100 bp

What went wrong? We eliminated the following possibilities through further testing

- Incorrect annealing temperature by using a temperature gradient
- Too much cDNA in the reaction by reducing the amount of cDNA from micrograms to ~100 nanograms/ 50 uL reaction



One Important Question!

Was there mRNA coding for Fraa1 in the strawberries when we extracted mRNA?

Further research on Fraa1 reveals that Fraa1 is part of the ripening mechanism of strawberries

Strawberries are non-climactic fruit, which stop all ripening metabolic processes after removal from plant. Our strawberries were store-bought, and therefore have been “dead” for at least two or three days, giving the mRNA coding for Fraa1 plenty of time to degrade.

Next experiment ----

- Make sure that we have reason to believe that our sample is in a state that contains the appropriate mRNA

Onward!

Search for allergens in arabidopsis

It should be ready(er) by now.

Namely:

LTP1 (documented allergen)
Bet v 1 (thaliana <= latera <= birch homologue via BLAST)
Others (list, links on wiki)

*Bet v 1: Pathogenesis-related proteins

*Lipid transfer protein

*Germin: stress protein

*Profilin: actin binding


cs 8773 3
9 vi 10


9 vi 10


Ca 8Y733
9 vi 10


Ca 87733
9 vi 10


50ug isn't much, but
neither are our plants!


We would feel much less guilty sampling plants of this size or larger.
More importantly, sampling should be more reliable.

They should be larger by now.

After all, they spent the weekend happily growing in the underground greenhouse bunker.

Future Directions

Identify Allergens

Design Primers for shRNA

PCR out our intron, antisense, sense sequences

amiRNA ?

Clone into Biobrick vector (VO210)

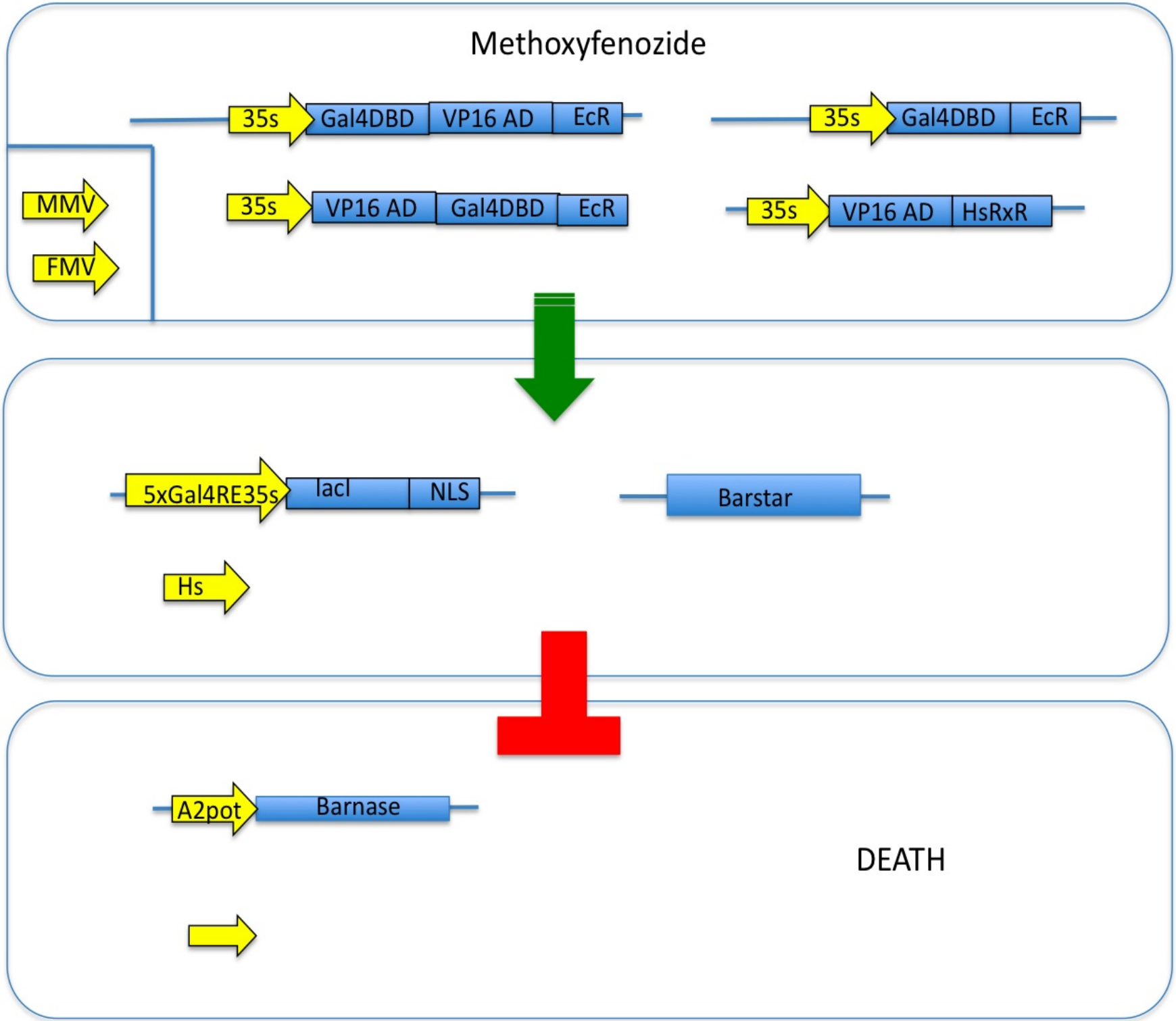
Transform into E. coli

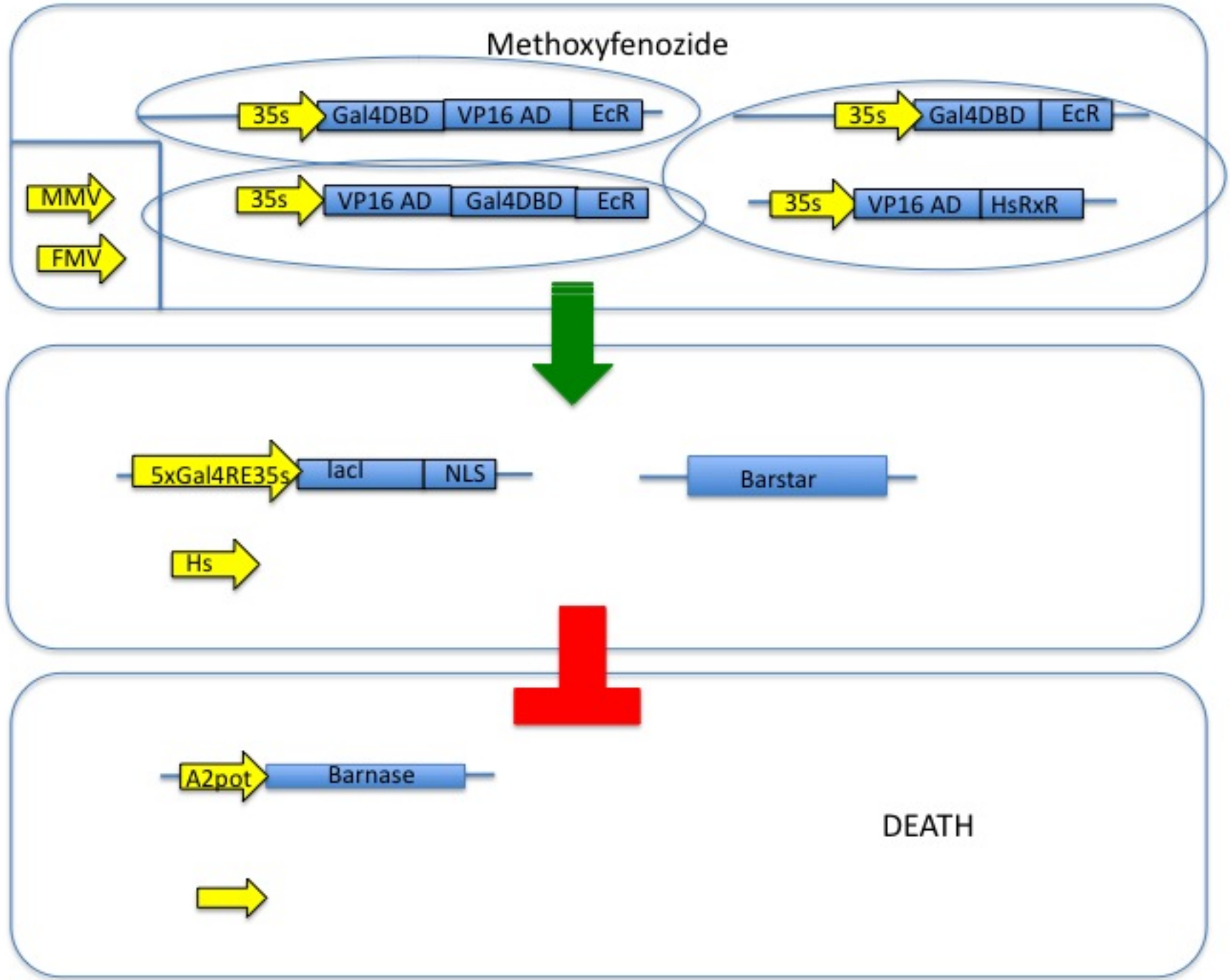
Insert into agrobacterium

Transform into Arabidopsis/ Plants

Observe Allergen Knockdown

TEAM GENETIC FENCE


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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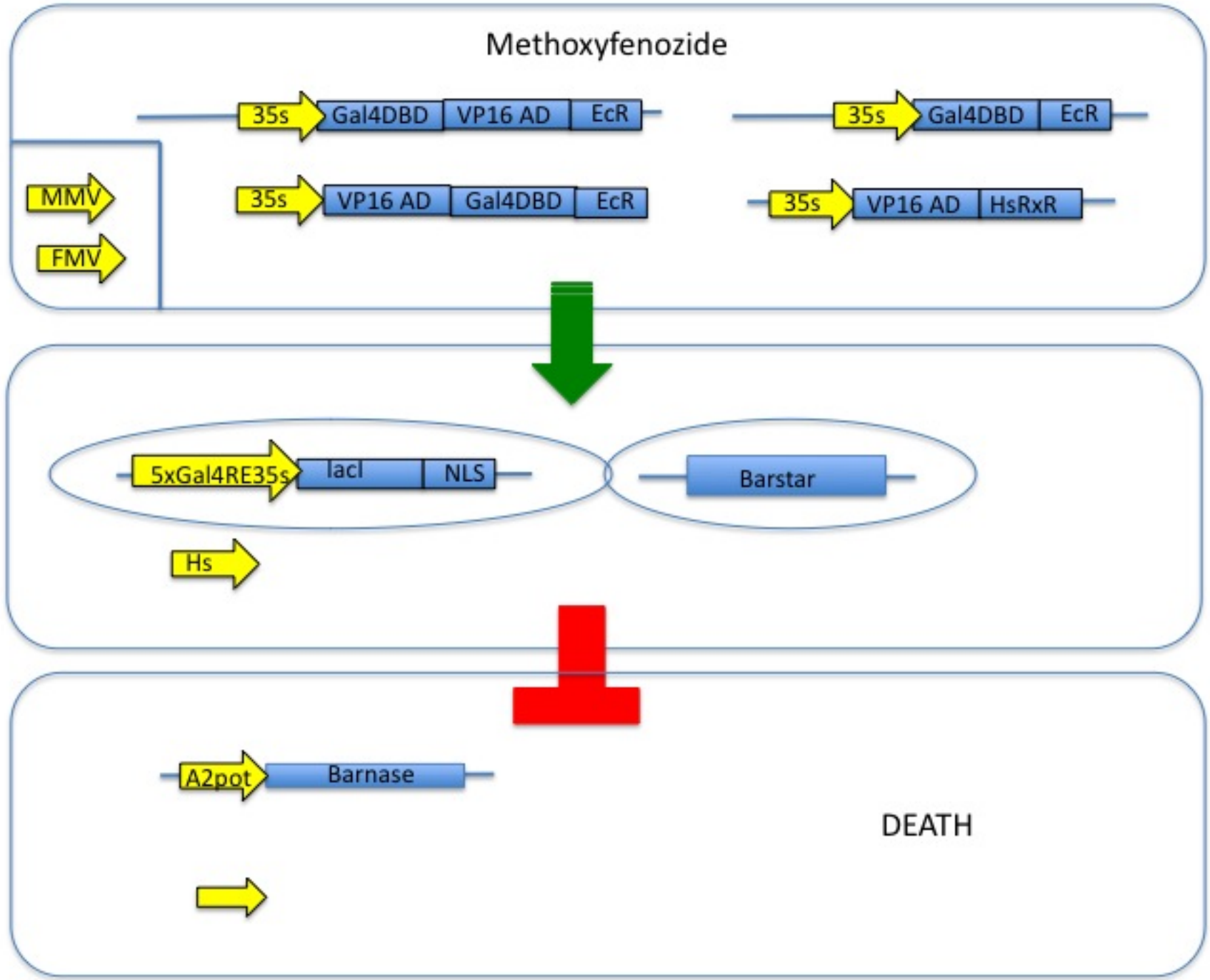
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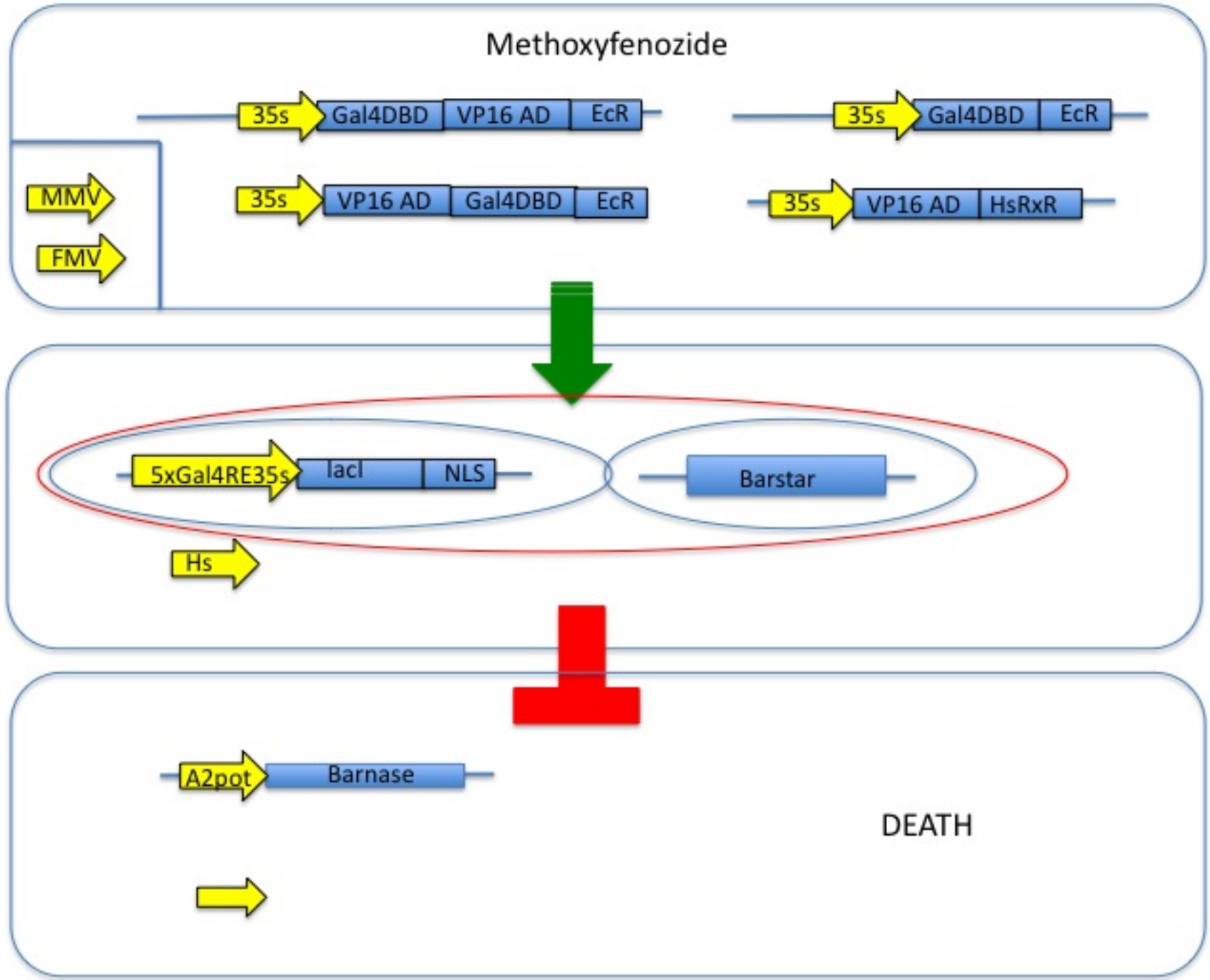
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VP16 AD
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Gal4DBD
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Gal4DBD
EcR
35s
VP16 AD
HsRxR
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lacI
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Hs
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Barnase
DEATH

Last Week

- Methoxyfenozide was ordered and arrived Thursday
- Made requests to other labs for LacI with NLS, ActIn promoter with lacO sites (A2pot), and Cassava Vein Mosaic Virus promoter, as well as plasmids containing the entire monopartate and bipartate switch mechanisms
- Barstar and Barnase ordered from ADDGENE

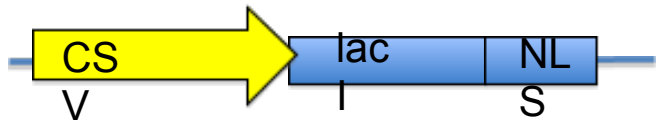
Last Week, Cont.

- VP16, GAL4 DNA Binding Domain, and LacI in biobricks registry
- LacI
  - Two biobricks in registry, one wildtype and one including and LVA rapid degradation tail
  - Transformed, minipreped and digested both LacI and LacI+LVA
- VP16
  - Transformed, minipreped and digested VP16
- GAL4 DBD
  - Transformed
- One run of PCR to attach NLS to LacI
  - Also annealed oligos to form NLS alone for biobrick

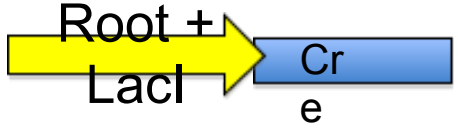
Alternate/Secondary System: Crelox and IPTG

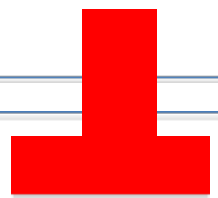
- Attractive paradigm (lactose analog instead of pesticide)
- However untested and relies on less-characterized parts

Lac +
Cre/Lox



Root +
LacI





TECHNICAL ADVANCE

External control of transgene expression in tobacco plastids using the bacterial *lac* repressor

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Received 17 March 2005; revised 9 June 2005; accepted 17 June 2005.
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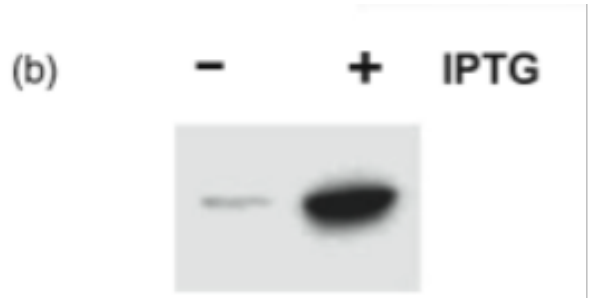
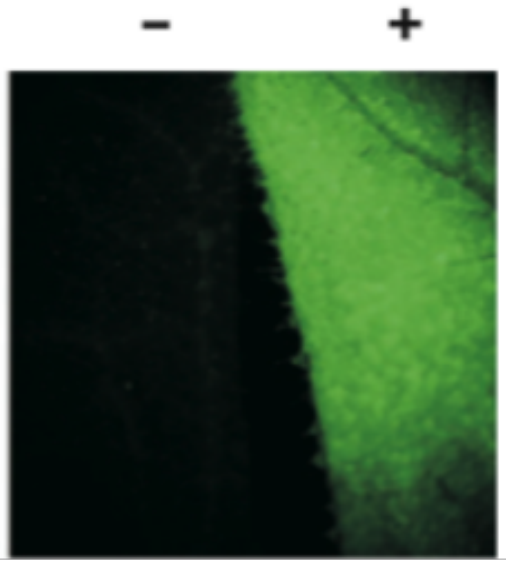
Summary

Although several induction systems have been described for plants containing transgenes in the nucleus, to date there is only one method for controlling transgene expression in plastids. This consists of chemical induction of a nuclear gene and import of the gene product into plastids, so that transformation of two cellular compartments is required. Here we describe a system for external control of plastid gene expression which is based entirely on plastid components and can therefore be established in a single transformation step. Our system uses modified promoters containing binding sites for the bacterial *lac* repressor. Chemical induction can be made with intact plants or after harvesting, which provides ecological and economic benefits.

Keywords: plastid transformation, transgene expression, induction, *lac* repressor, IPTG.





(a)

-35 -10

PrrnP1: ..tggataagaggctcgtgggattgacgtgaggggcagggatggctatatttctgggagcgaactccgggcg..

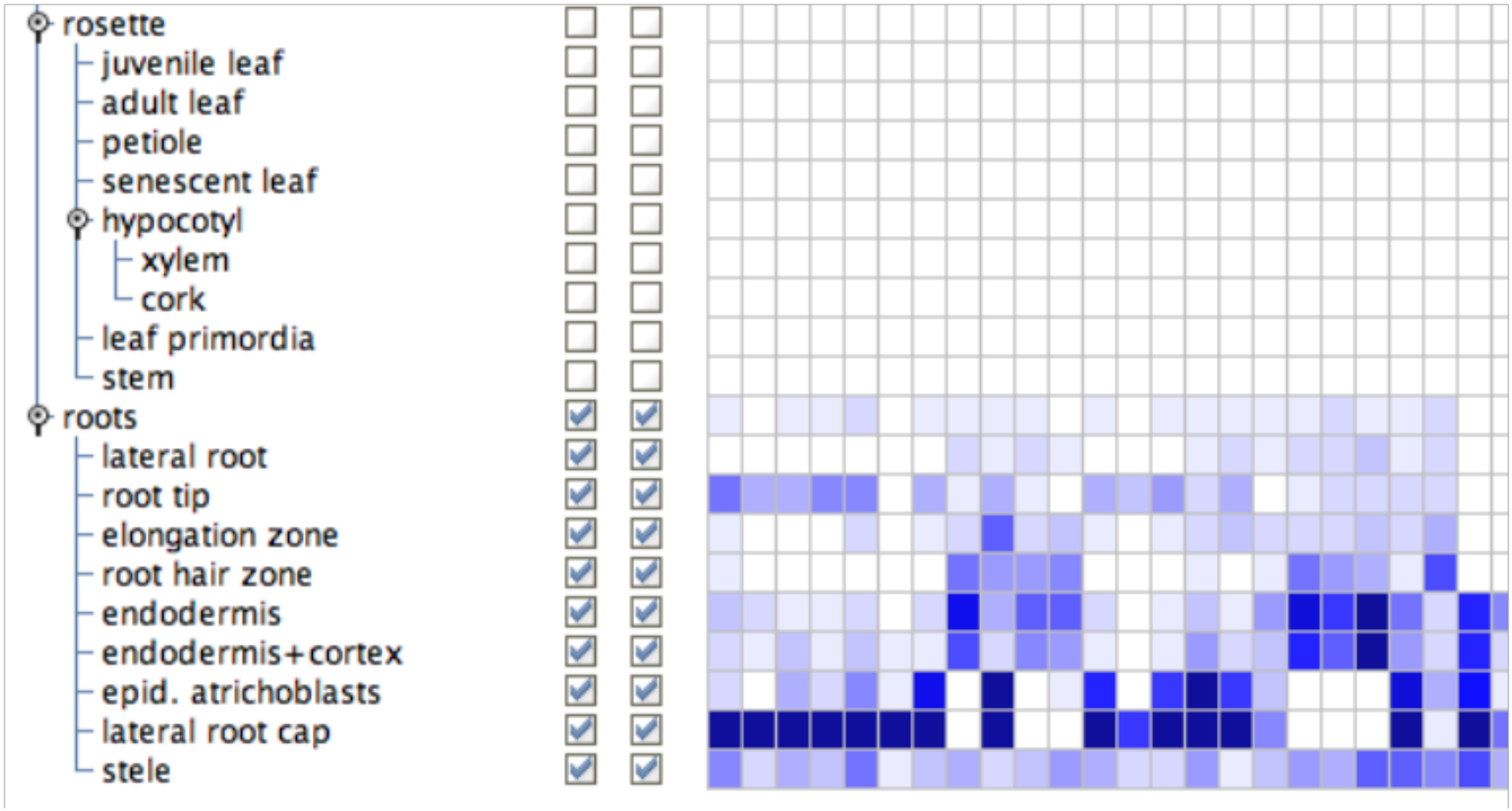
Prrnlac1: ..tggataagaggctcgtgggattgacgtgaggggcagggatggctatatttctggg**attgtgagcggataacaa**g..

Prrnlac2: ..tggataagaggctcgtgggattgacg**attgtgagcggataacaa**tatatttctgggagcgaactccgggcg..

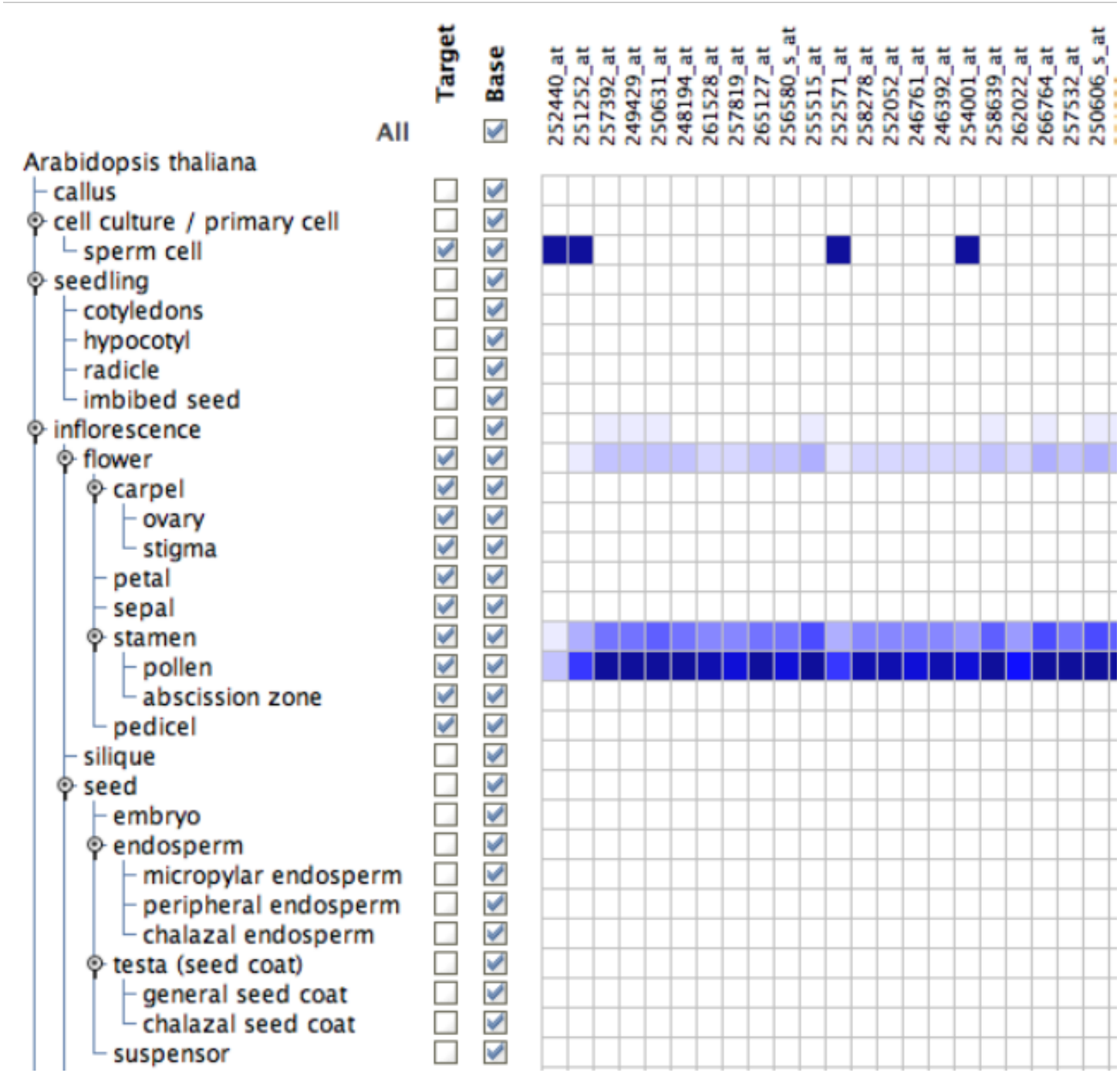
Prrnlac3: ..tggataagaggctcgtgggattgacg**attgtgagcggataacaa**tatatttctggg**attgtgagcggataacaa**g..

Plac: ..*actcattaggcaccccaggctttacactttatgcttccggctcgtatgttgtgtgga***attgtgagcggataacaa**t..

Tissue Specific Promoters



Back up:


All
Target
Base
252440_at
251252_at
257392_at
249429_at
250631_at
248194_at
261528_at
257819_at
265127_at
256580_s_at
255515_at
252571_at
258278_at
252052_at
246761_at
246392_at
254001_at
258639_at
262022_at
266764_at
257532_at
250606_s_at
Arabidopsis thaliana
callus
cell culture / primary cell
sperm cell
seedling
cotyledons
hypocotyl
radicle
imbibed seed
inflorescence
flower
carpel
ovary
stigma
petal
sepal
stamen
pollen
abscission zone
pedicel
silique
seed
embryo
endosperm
micropylar endosperm
peripheral endosperm
chalazal endosperm
testa (seed coat)
general seed coat
chalazal seed coat
suspensor

Other considerations

- Plastid DNA and Chloroplast modification
  - dramatically reduces risk of cross-pollenation
  - Increases technical difficulty of transformation (agrobacterial vector no longer sufficient

- Time specific tissue-specific promoters for cre-lox?