Cloning and Expression of a Bacterial CGTase and Impacts on Phytoremediation

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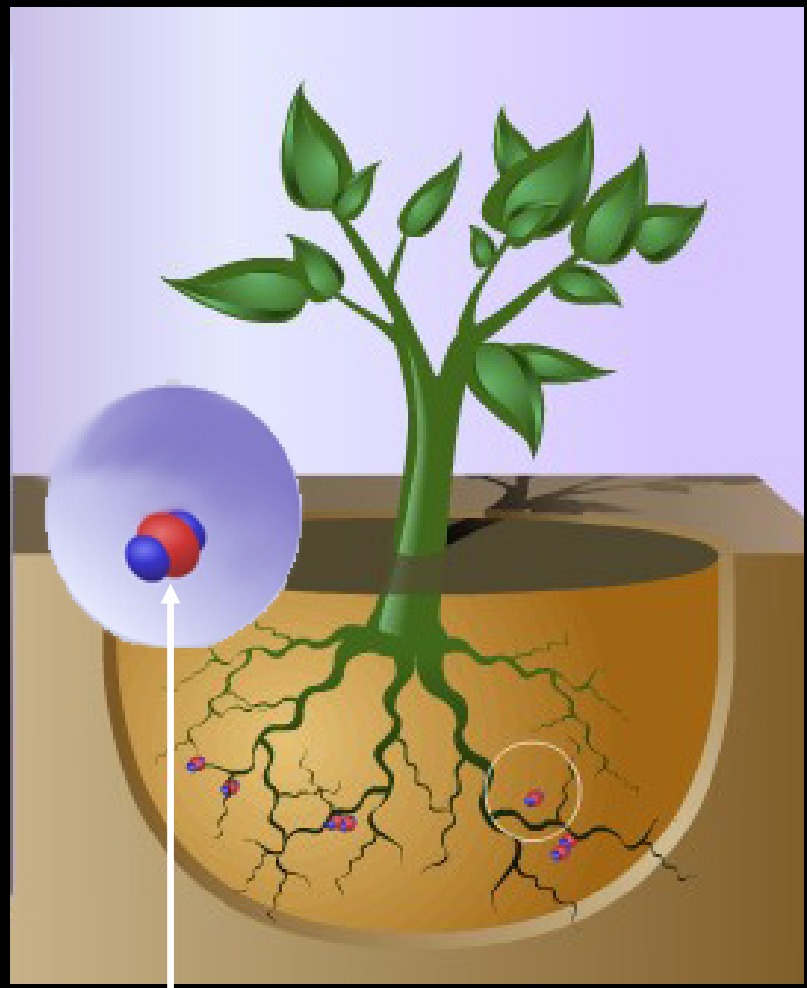
Phytoremediation of Organic Compounds

Phytodegradation:

Plants directly degrade contaminants using their own Enzymatic processes

Rhizosphere assisted Phytoremediation:

Plants provide carbon source, stable environment for Bacterial degradation



Bioavailability:

Are the contaminants accessible to plants and bacteria?

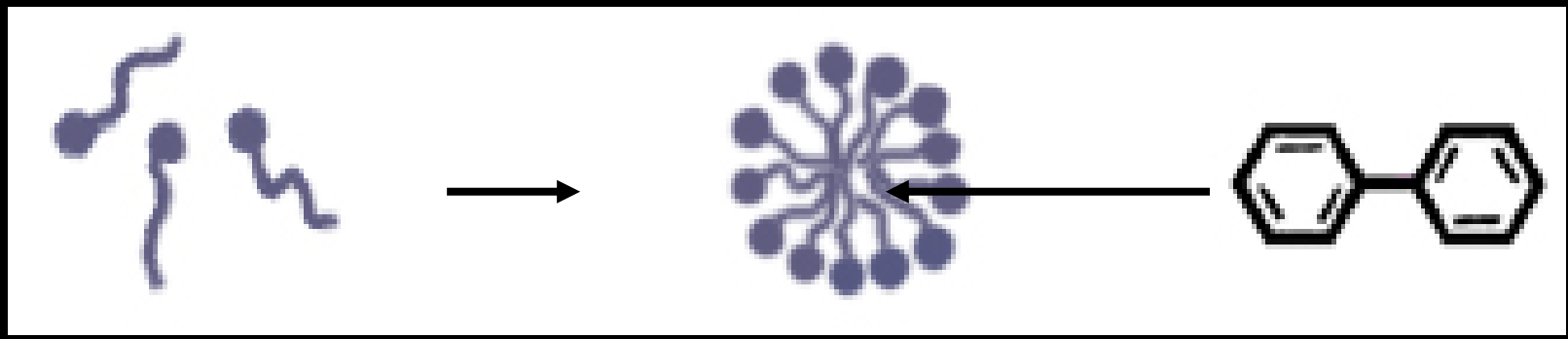
Contaminants may be sufficiently available to cause damage

But not sufficiently available for effective biological remediation

Lack of bioavailability is one of the major limitations on phytoremediation of Persistent Organic Pollutants (POPs)

Surfacants:

Proposed as a means of overcoming
Bioavailability limitations on phytoremediation



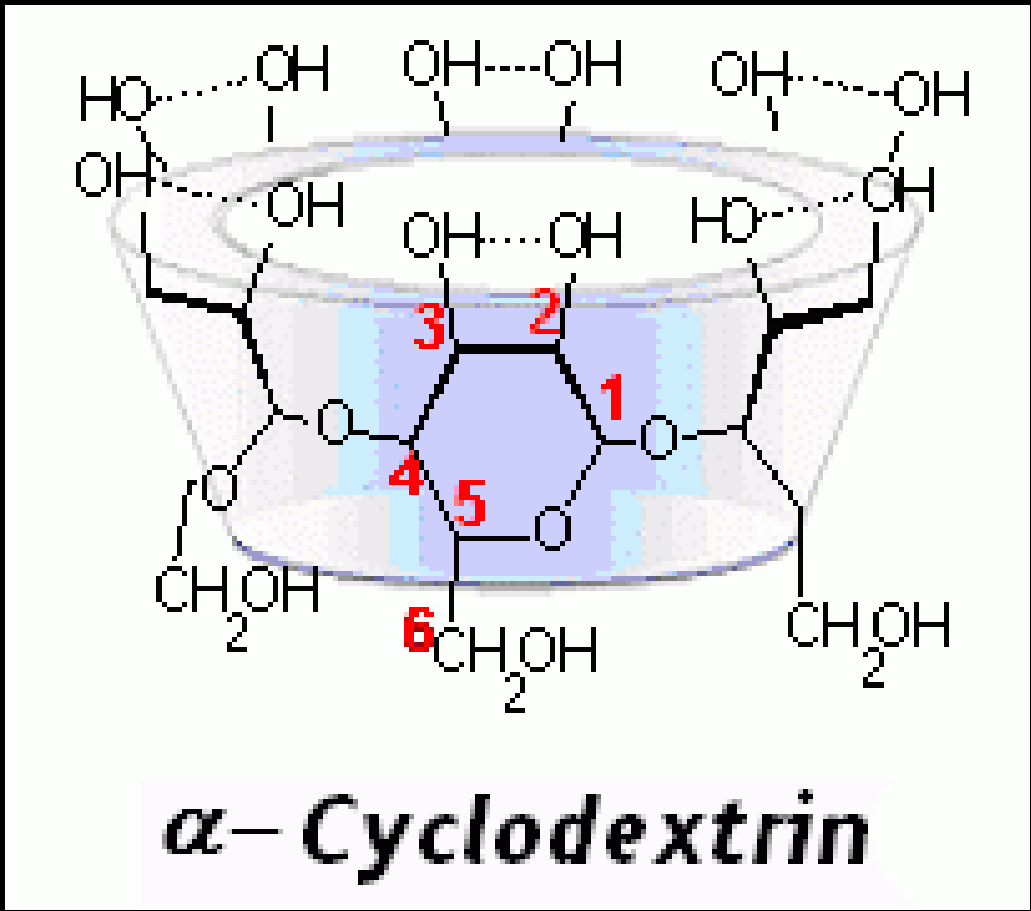
Surfactant Monomers Surfactant Micelle Organic compound

-Surfactants can increase apparent water solubility of contaminants in soil

- May cause bacterial toxicity

- Maximum effectiveness at critical micelle concentration

Cyclodextrins

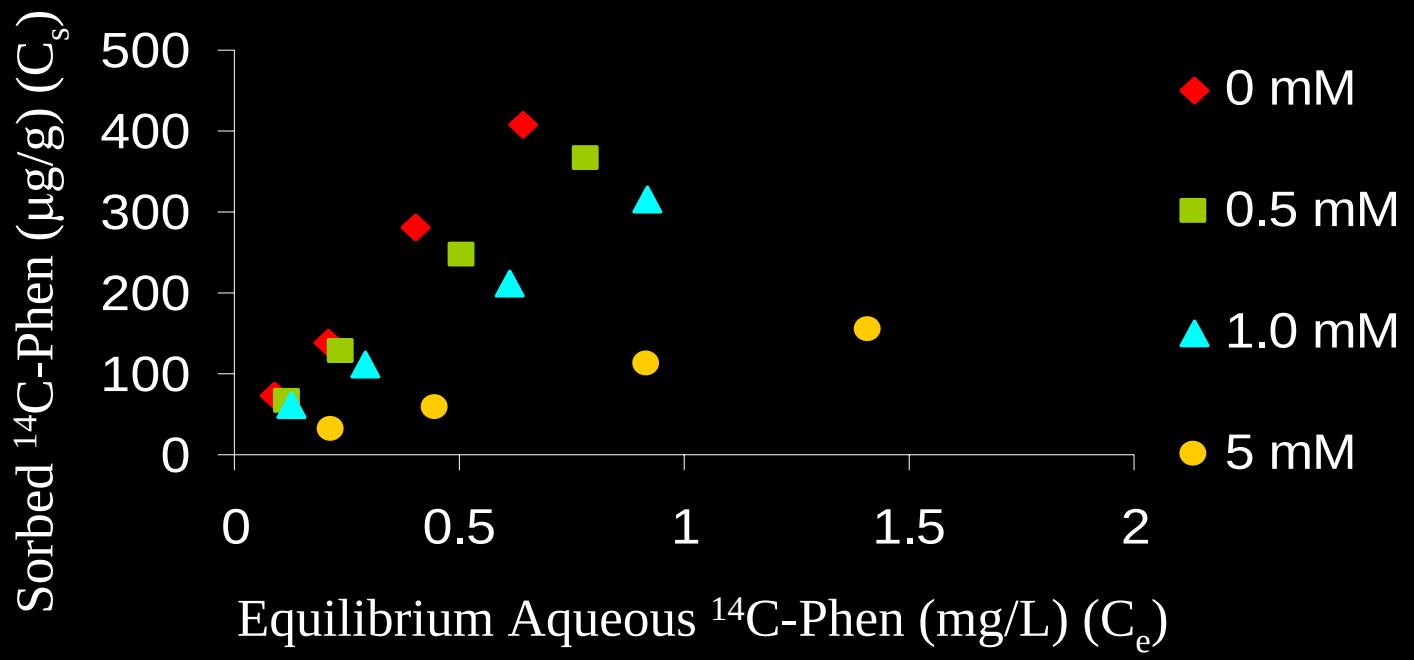


- Cyclic compounds composed of 6-8 glucose units
- Solubilizing properties similar to surfactants
- Form stable complexes with organic compounds
- No critical micelle concentration
- Relatively non-toxic

Bioavailability: Potential for improvement

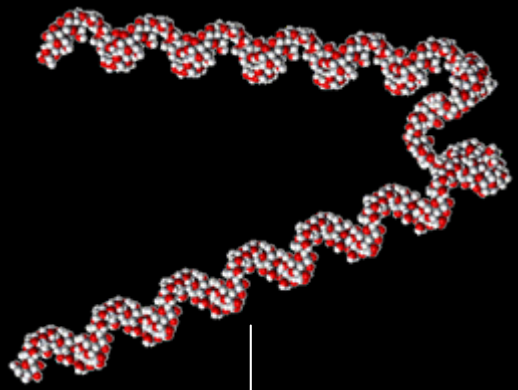
1. Cyclodextrins have been shown to improve the water solubility of various organic compounds

2. Cyclodextrins could increase bioavailability and enhance biological degradation.

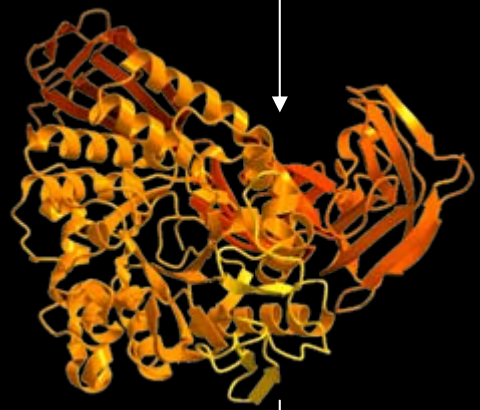


Cyclodextrin Glycosyltransferase (CGTase)

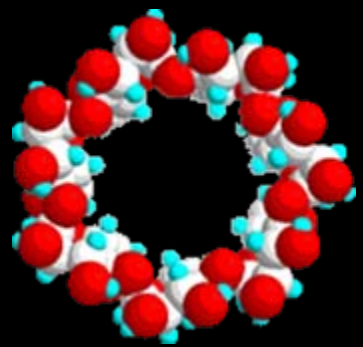
Starch



CGTase



Cyclodextrin



1. Degrades starch to cyclodextrin
2. Found in *Bacillus* and related species
3. Extracellular enzyme secreted into the environment

Methods for CD addition to soil

1. Direct addition
   - Can be expensive and time consuming

2. Addition of CGTase producers and starch
   - Strain persistence and *cgt* expression could be problematic

3. CGTase secretion by transgenic plants
   - More controllable and predictable
   - Other uses for plant-produced CGTase

Approach: Expression of CGTase in Transgenic Plants

1. Clone and characterize a *cgt* gene for potential production of cyclodextrin

2. Modify *cgt* gene for expression and secretion of CGTase from plant roots

3. Test transgenic plants for effectiveness in phytoremediation

Research Outline

I. *cgt* gene cloning and expression in bacteria

A. Cloning and sequence comparisons
B. Modification
C. Expression and functional analysis

II. *cgt* gene expression in plants

A. Modification for plant expression
B. Generation of Transgenic Plants
C. Plant expression and function of CGTase

III. *cgt* plant effects on PAH degradation

Part I: CGTase cloning and expression in Bacteria

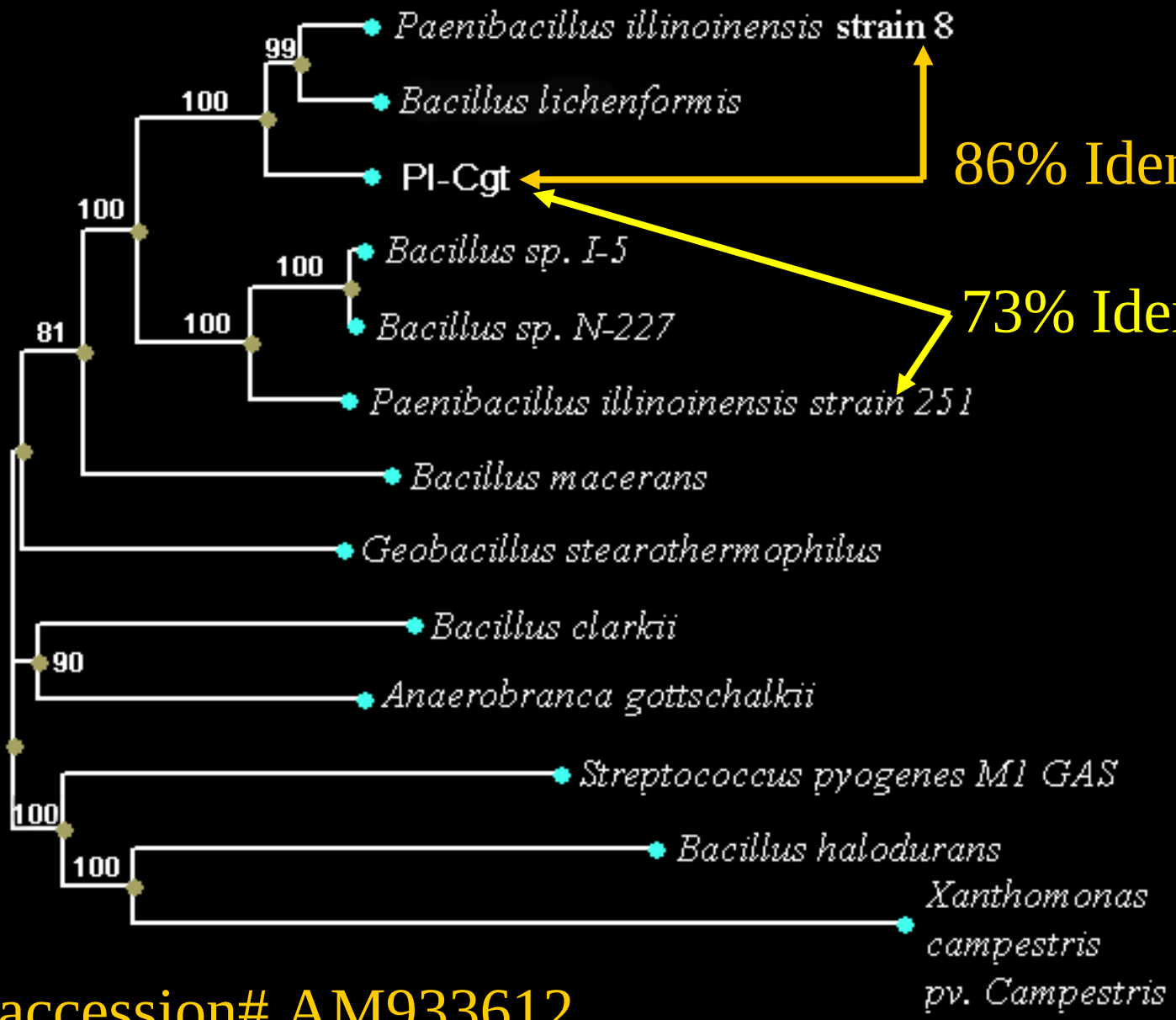
1. A novel gene was cloned from soil-isolated *Paenibacillus sp.*C36, designated PI-cgt

2. BLAST search reinforced potential identity of PI-cgt as a CGTase – highly similar to known CGTases

3. Phylogenetic tree was generated to determine the relationship of PI-cgt to other known CGTases

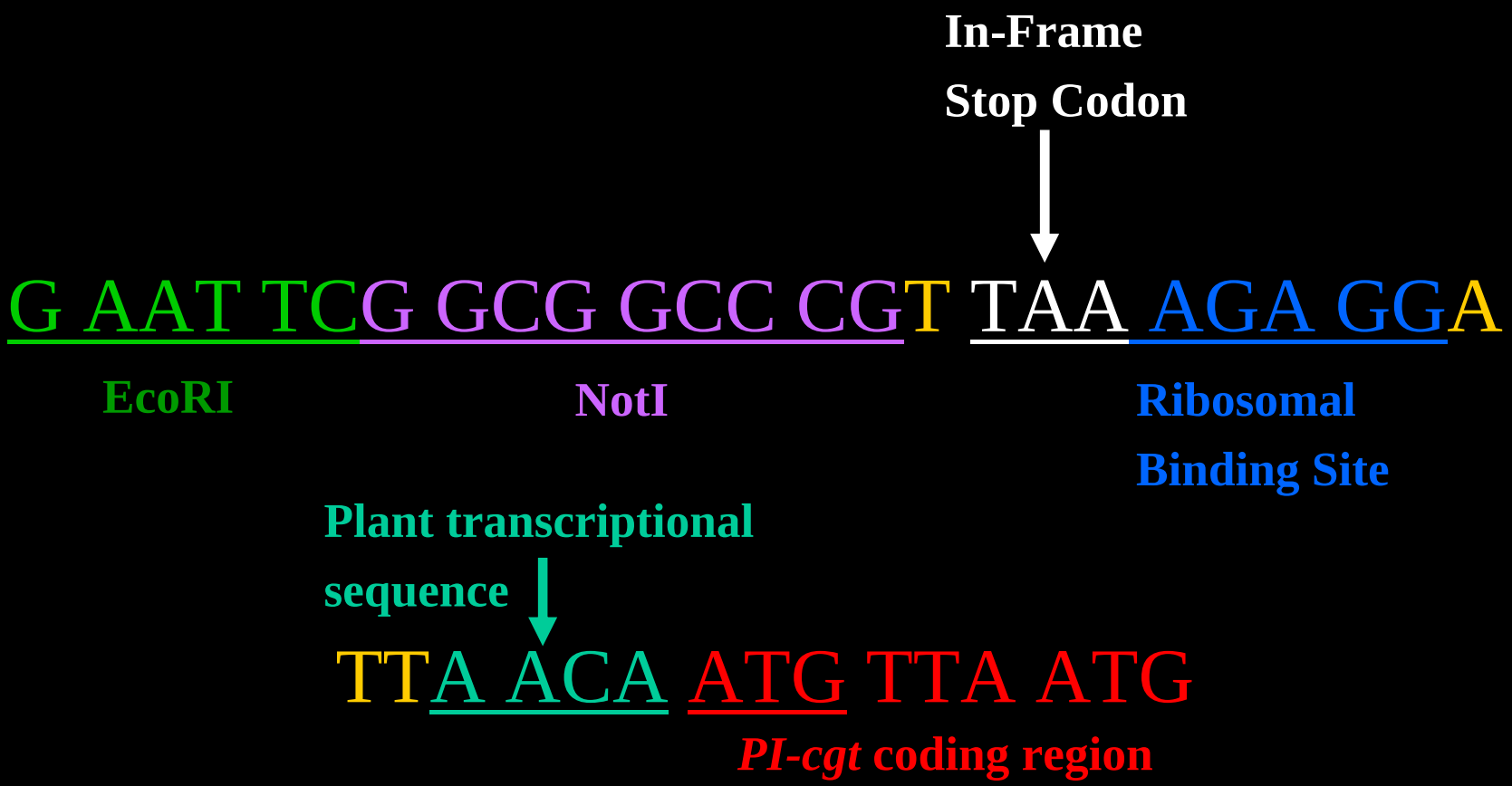
3. New primers were designed to allow expression of the novel gene in *E.coli*

Comparison to Known CGTases



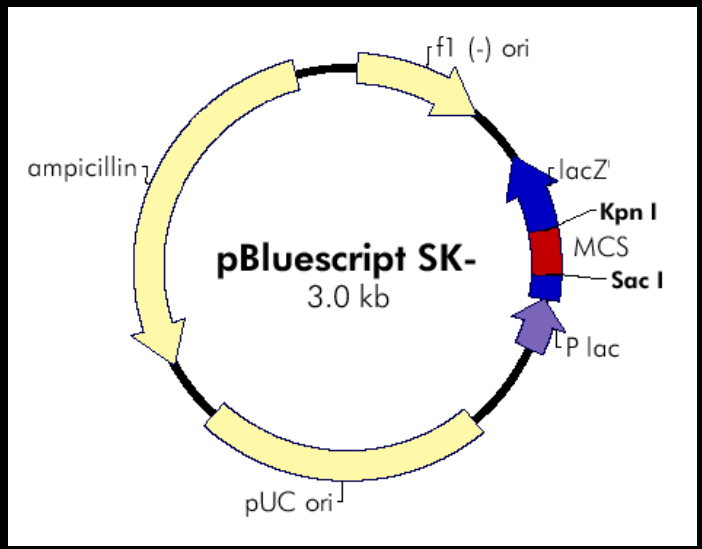
Genbank accession# AM933612

Modifications to the 5’ Coding region of *PI-cgt* for Bacterial and Plant expression

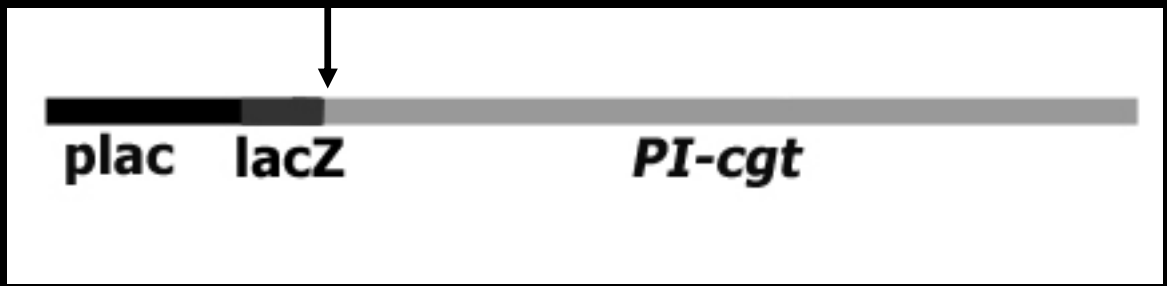


Expression of PI-cgt in *E. coli* DH5α

Signal peptides for extracellular secretion from gram positive bacteria should function in gram negative bacteria and eukaryotes as well



In-frame stop codon and Ribosomal Binding site



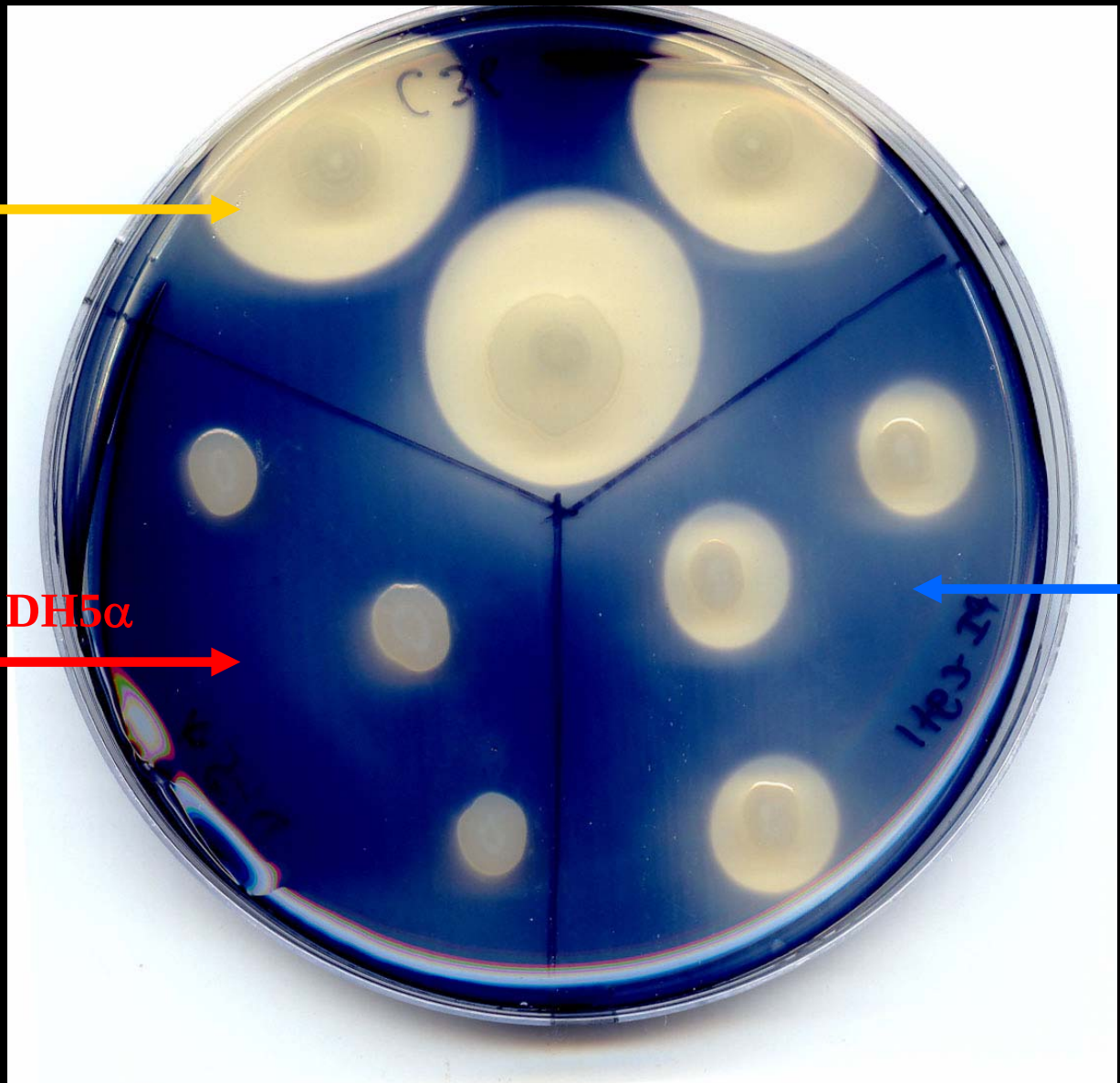
PI-cgt Functional Analysis

1. Clear zone formation on starch containing media – stained with iodine

2. Quantification of βCD production via phenolphthalein de-colorization

3. Qualitative examination of CD production via Thin Layer Chromatography

Bacterial Clear Zone production in Iodine stained Starch Media



***Paenibacillus* C36**

***E. coli* DH5α**

E. coli* pBluescript-*PI-cgt

Enzymatic Reaction for *in-vitro* Cyclodextrin Production

1-2 Day culture in basic medium or LB spun down

200µl 1.25% Starch
50µl supernatant

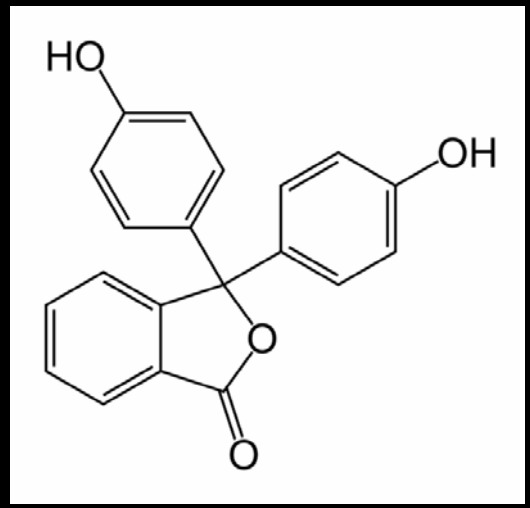
Incubate 1-3 hrs @ 55°C

Analyze using phenolphthalein reaction:

50µl sample
25µl phenolphthalein
20µl Na_2CO_3

Mechanism for Phenolphthalein reaction

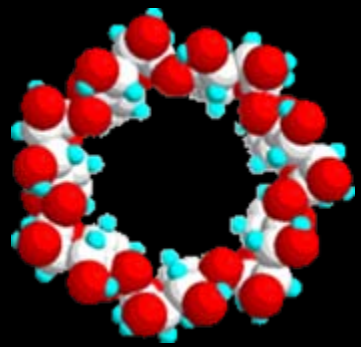
βCD, but not other CDs, forms a complex



Phenolphthalein (pink)

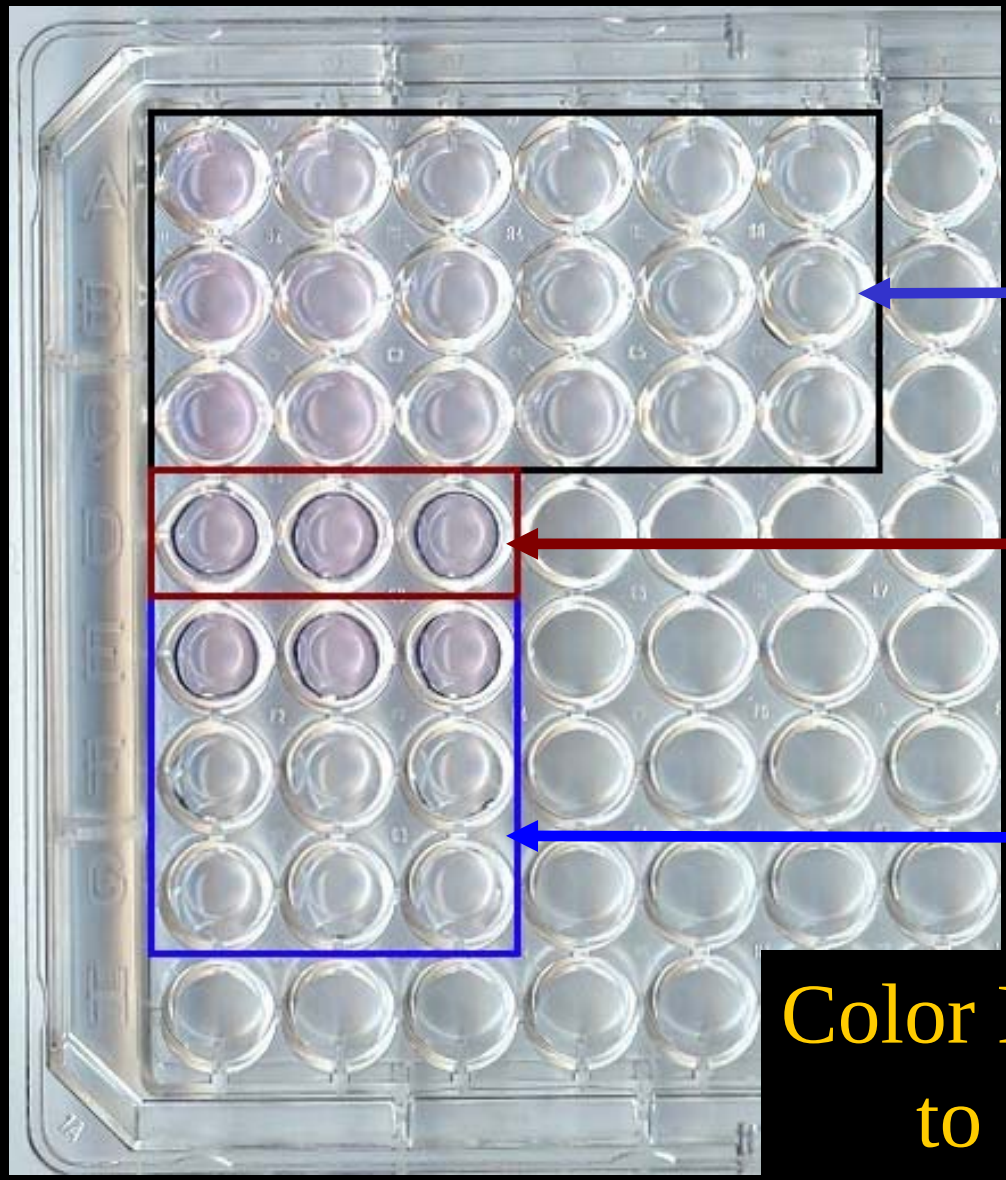
Complexed Phenolphthalein is de-colorized

Proportional to βCD content



βCD (de-colorizer)

Phenolphthalein βCD analysis



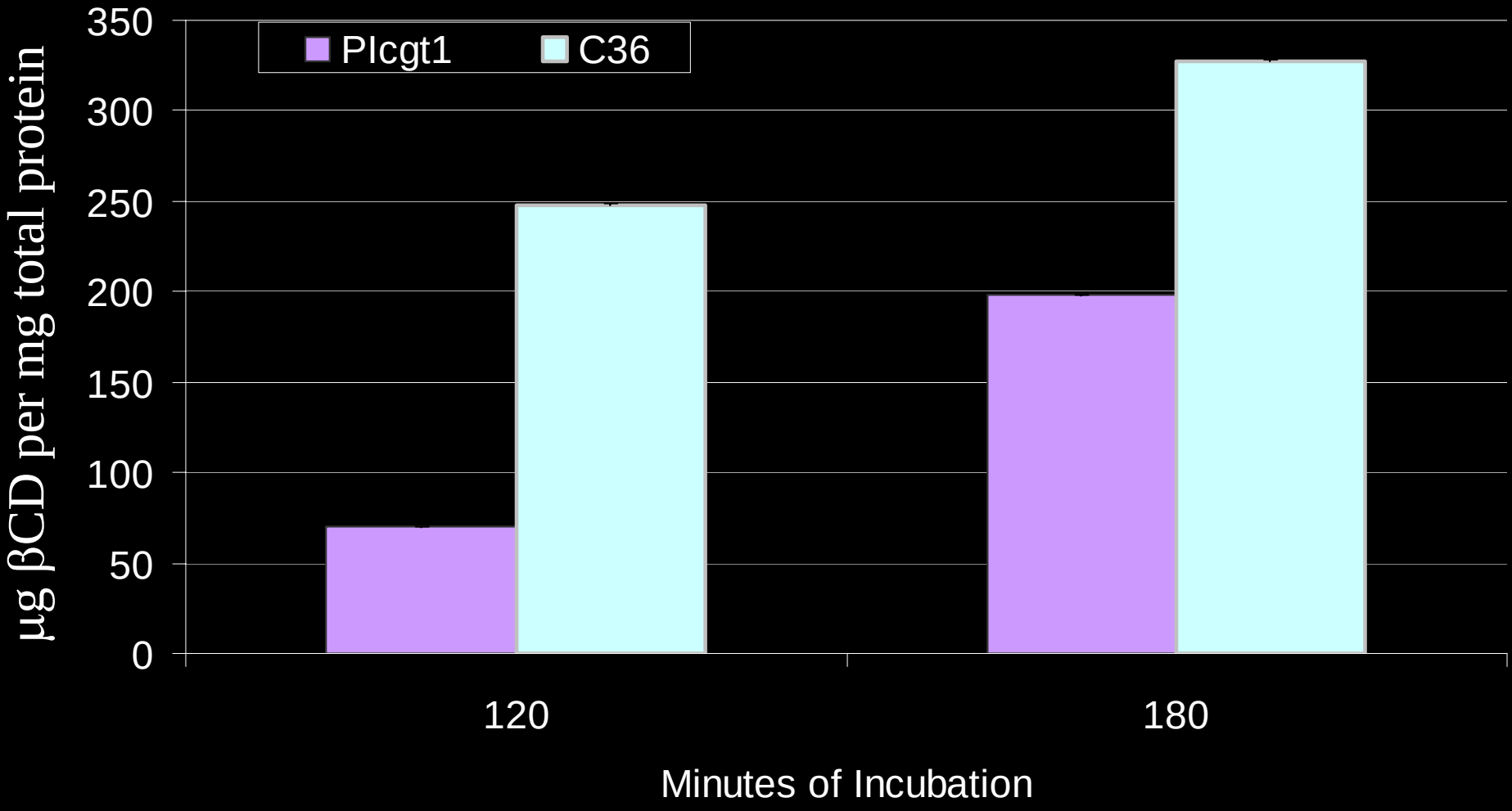
Standards

Wild Type

Cgt-Transformed Lines

Color Reduction proportional to βCD concentration

Bacterial βCD Production



Thin Layer Chromatographic analysis of Cyclodextrins

2μl spotted 4 times, mobile phase was acetonitrile-water-ammonium hydroxide (6:3:1)
Sprayed with Vaugh's solution
Developed using a hot plate.

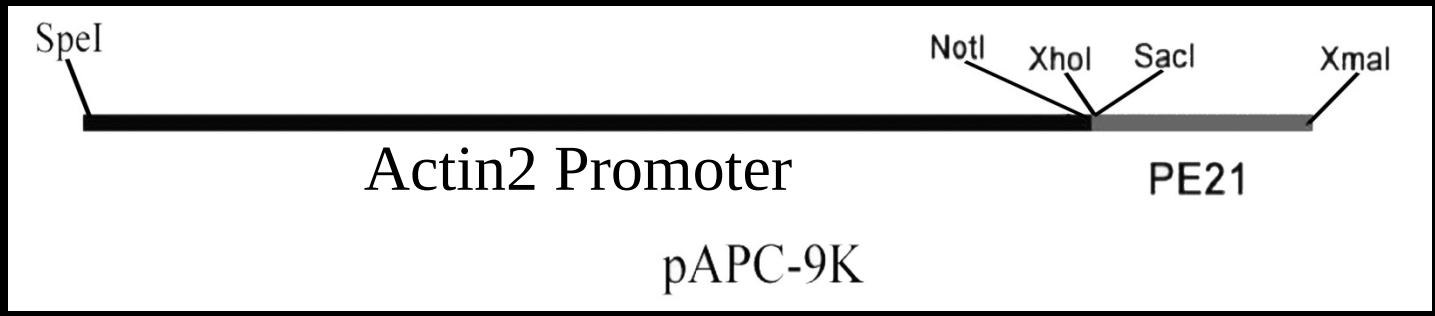
Part II: CGTase expression in plants

1. PI-cgt was placed into plant expression vectors under the control of the Actin2 and “Super” promoters from Arabidopsis and Agrobacterium respectively

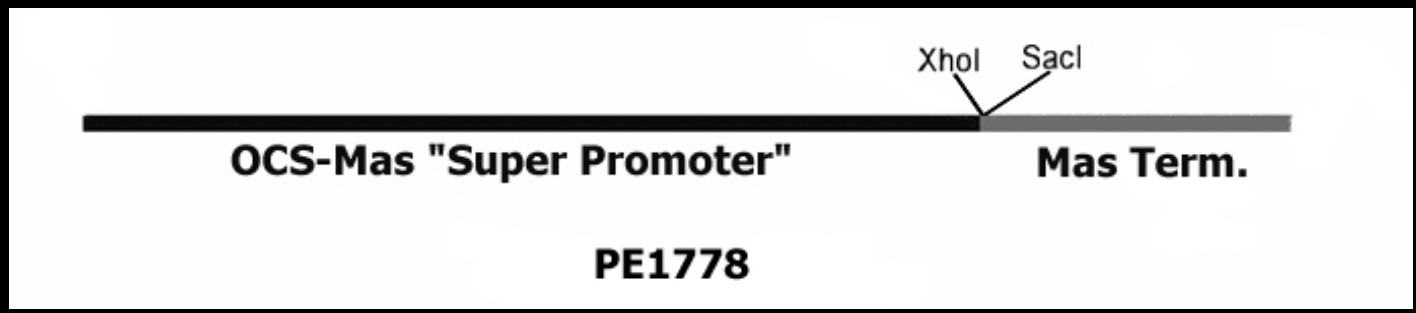
2. Transgenic plant lines were generated in tobacco and Arabidopsis

3. Plant expression was assayed using RT-PCR, starch clearing, and βCD production using phenolphthalein and TLC analysis

Constructs for expression in plants



Actin2 promoter was used for both tobacco and Arabidopsis, antibiotic marker was hygromycin (pC lines)



Super promoter was used only for tobacco, antibiotic marker was kanamycin (pE lines)

Plant Transformation

Arabidopsis was transformed via vacuum infiltration

Seed lots
Primary
Transformants

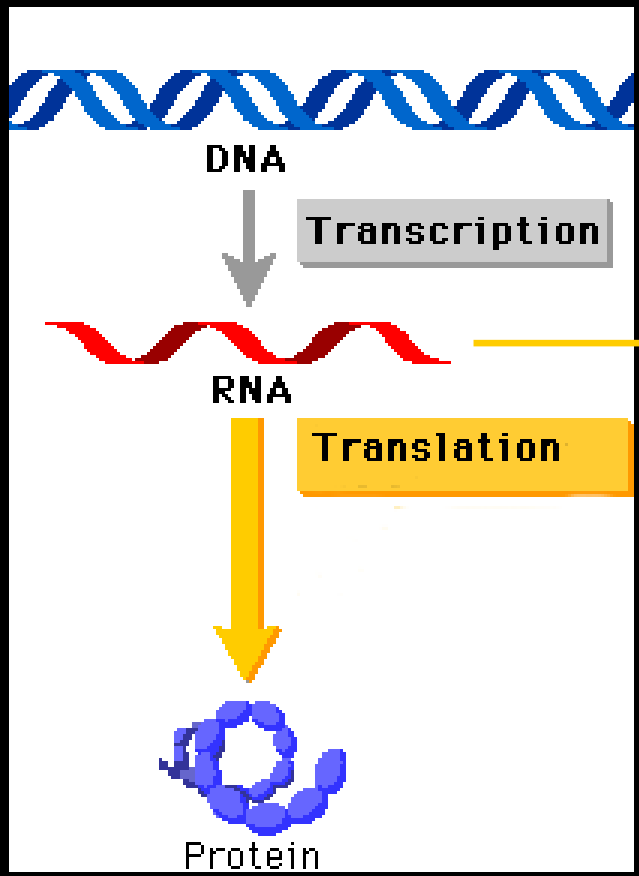
Tobacco transformed via co-cultivation

Single Plants
Primary
Transformants

PCR screening of *cgt* plant lines

Genomic PCR

Tests for PI-*cgt* integration into the plant genome



Reverse Transcriptase PCR (RT-PCR)

Tests for PI-*cgt* expression: Production of mRNA

RT-PCR screening of Putative Transgenic Arabidopsis Plants for *PI-cgt* expression

cgt primers

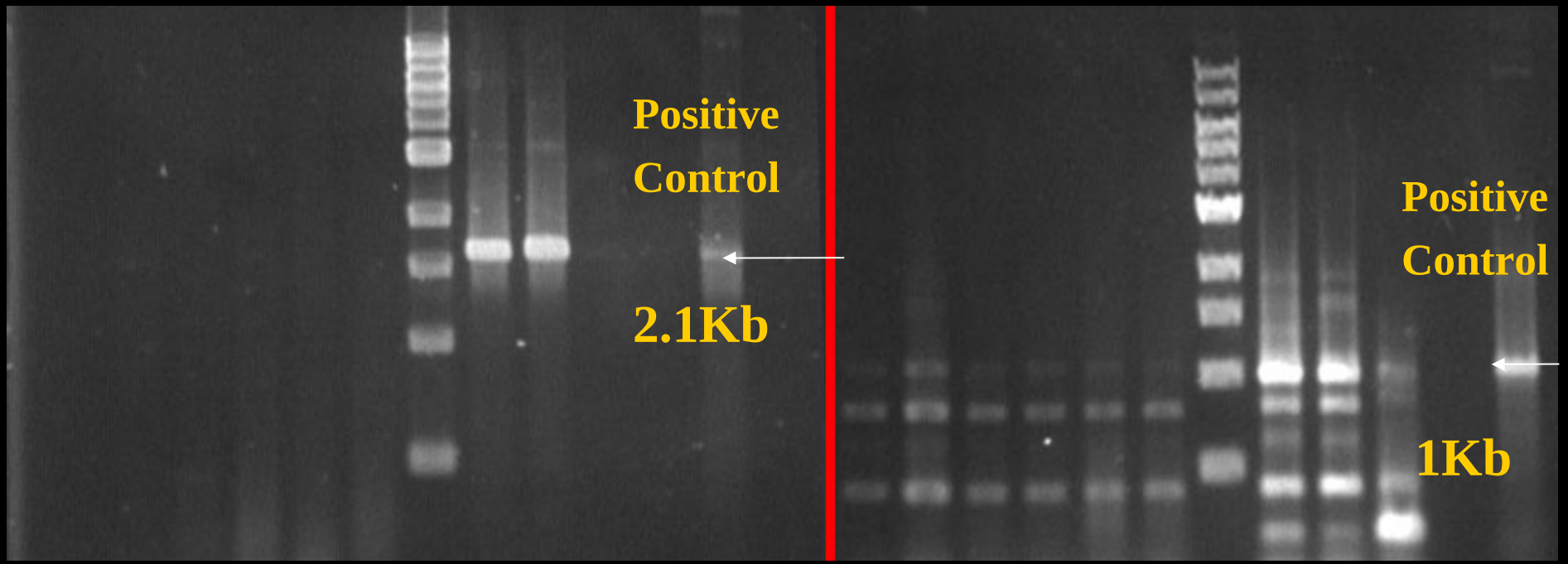
RNA controls

cgt cDNA

hyg primers

RNA controls

cgt cDNA



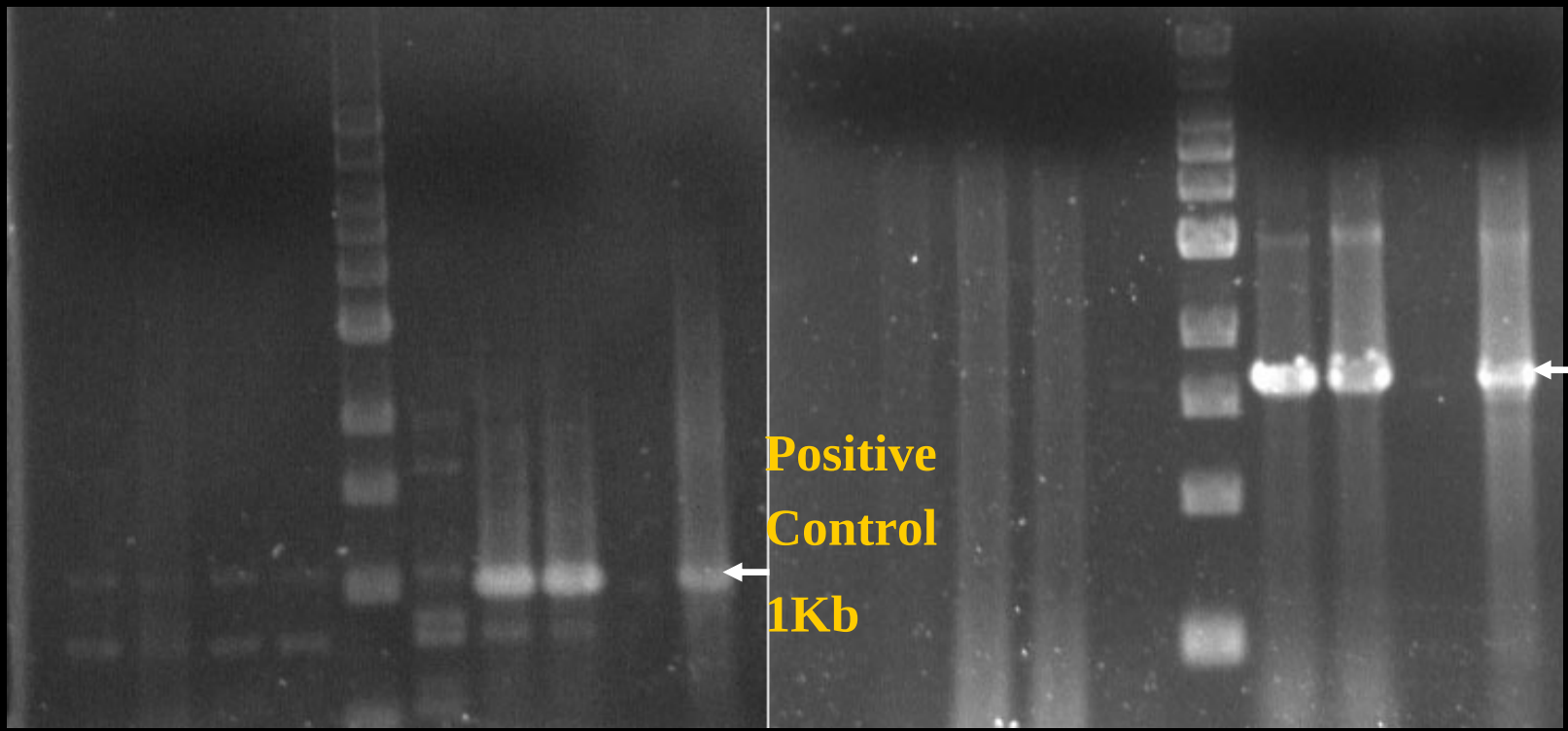
RT-PCR screening of Putative Transgenic Tobacco Plants for *PI-cgt* expression

RNA controls PC lines cDNA

hyg primers

RNA controls PC lines cDNA

cgt primers



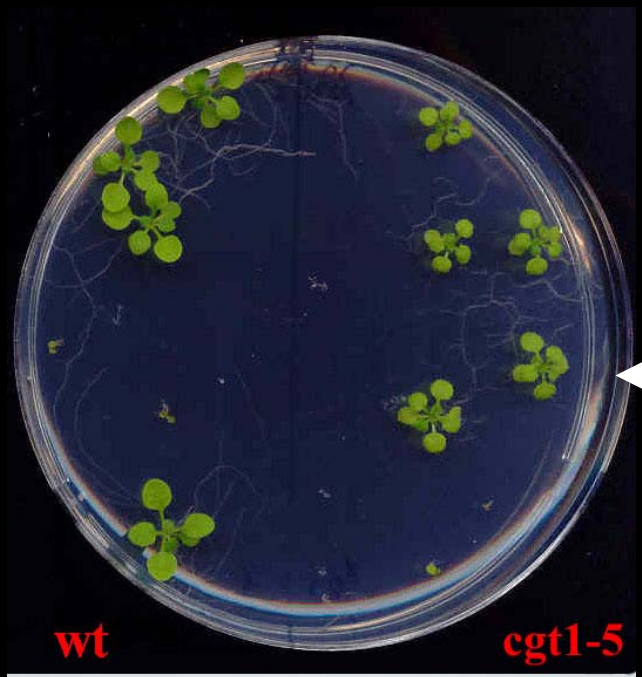
PI-cgt Functional Analysis in Plants

1. Clear zone formation on starch containing media – stained with iodine

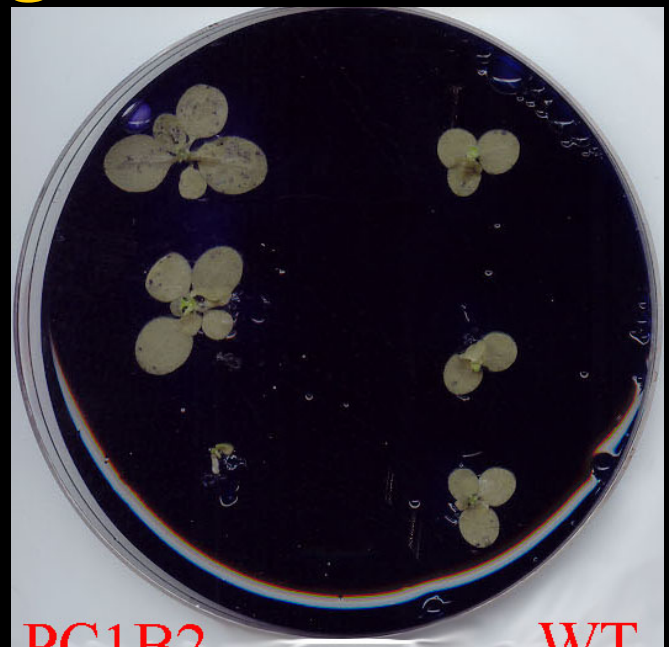
2. Quantification of βCD production via phenolphthalein de-colorization

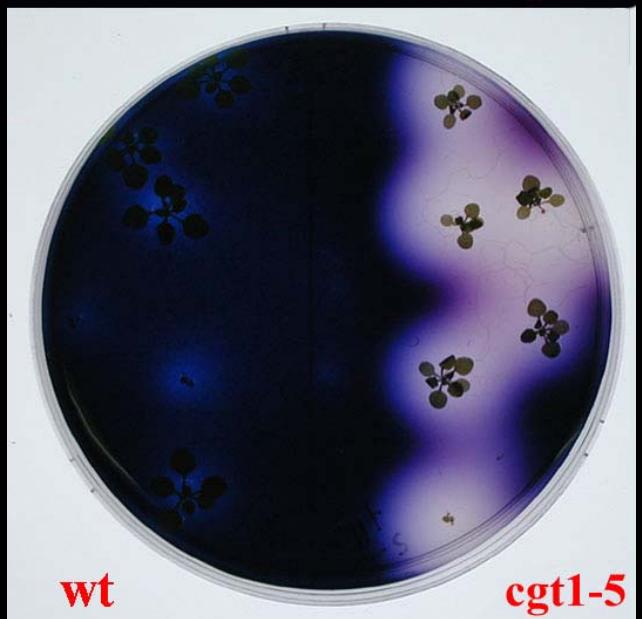
3. Qualitative examination of CD production via TLC

Starch Clearing by Transgenic Plants

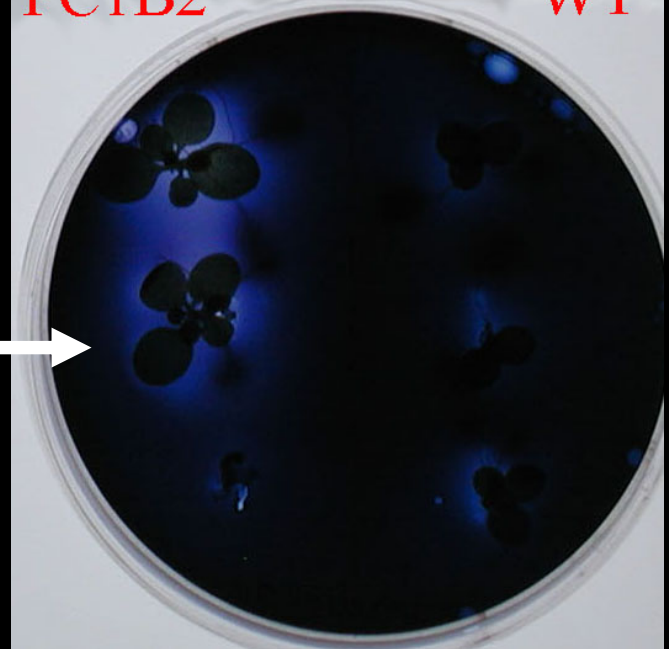


Arabidopsis



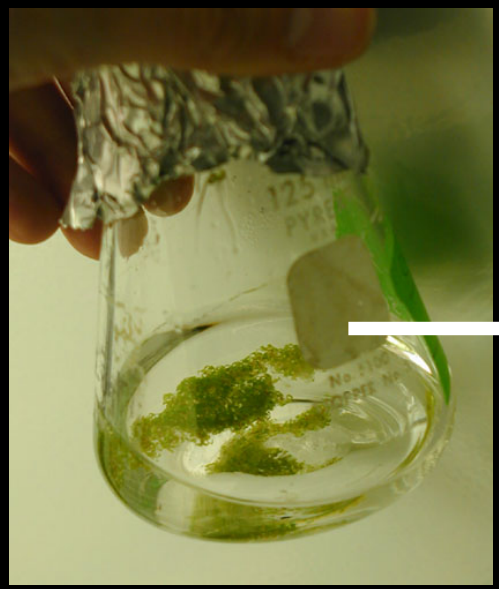


Tobacco

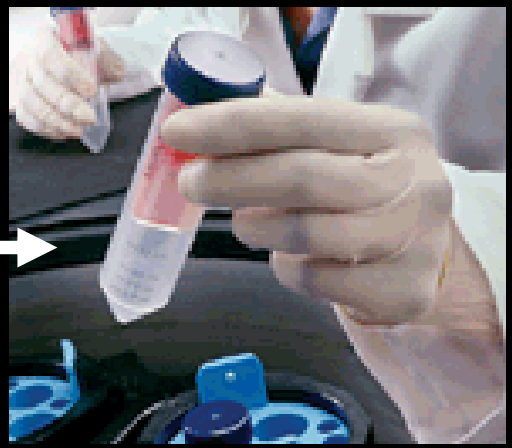


In-vitro CGTase production

Seedlings grown Hydroponically



Centrifugal concentration



200µl of 1.25% Starch

50µl of concentrate

Incubate 1-3 days @ 55°C

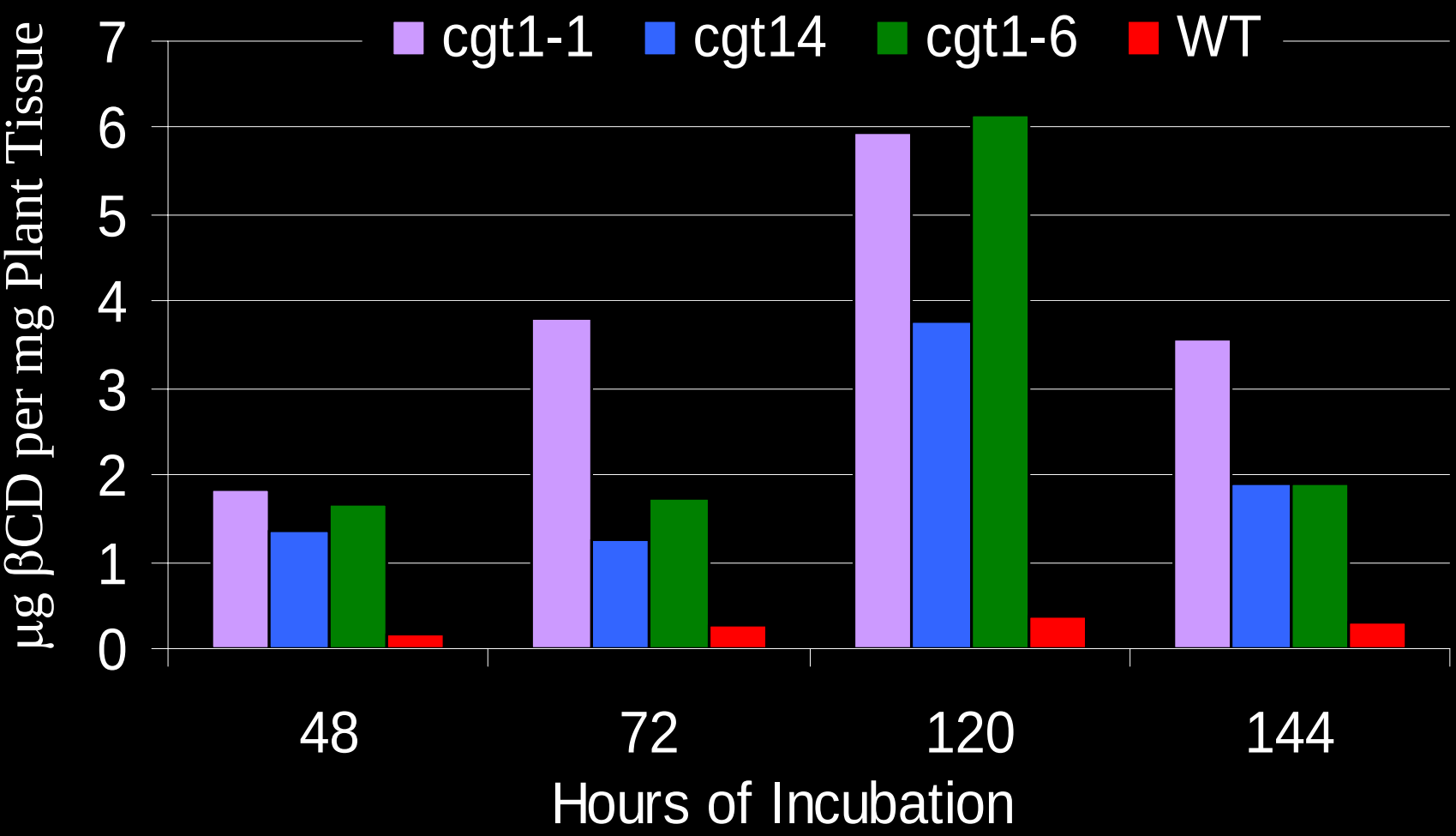
Analyze using phenolphthalein reaction:

50µl sample

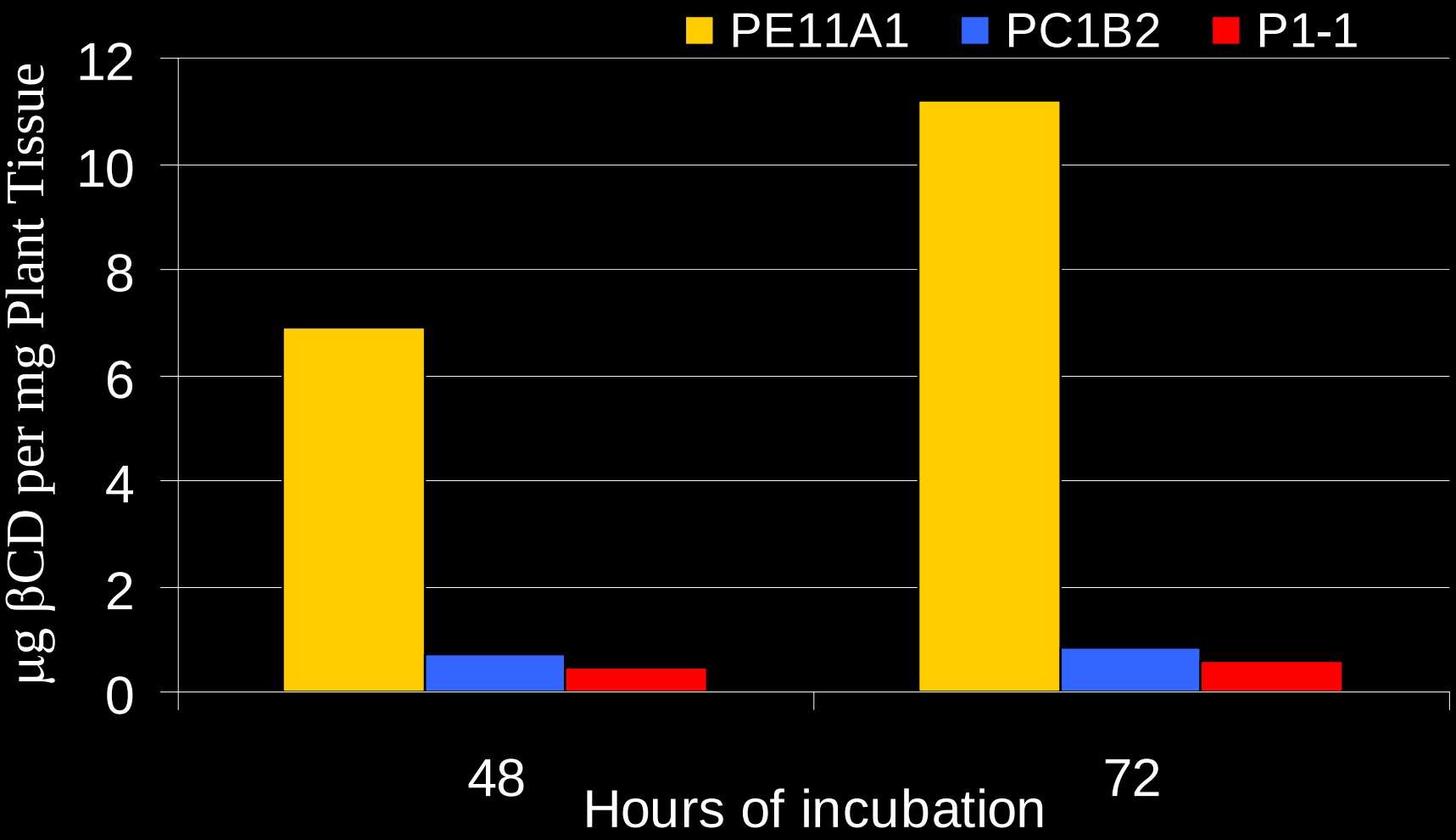
25µl phenolphthalein

20µl Na_2CO_3

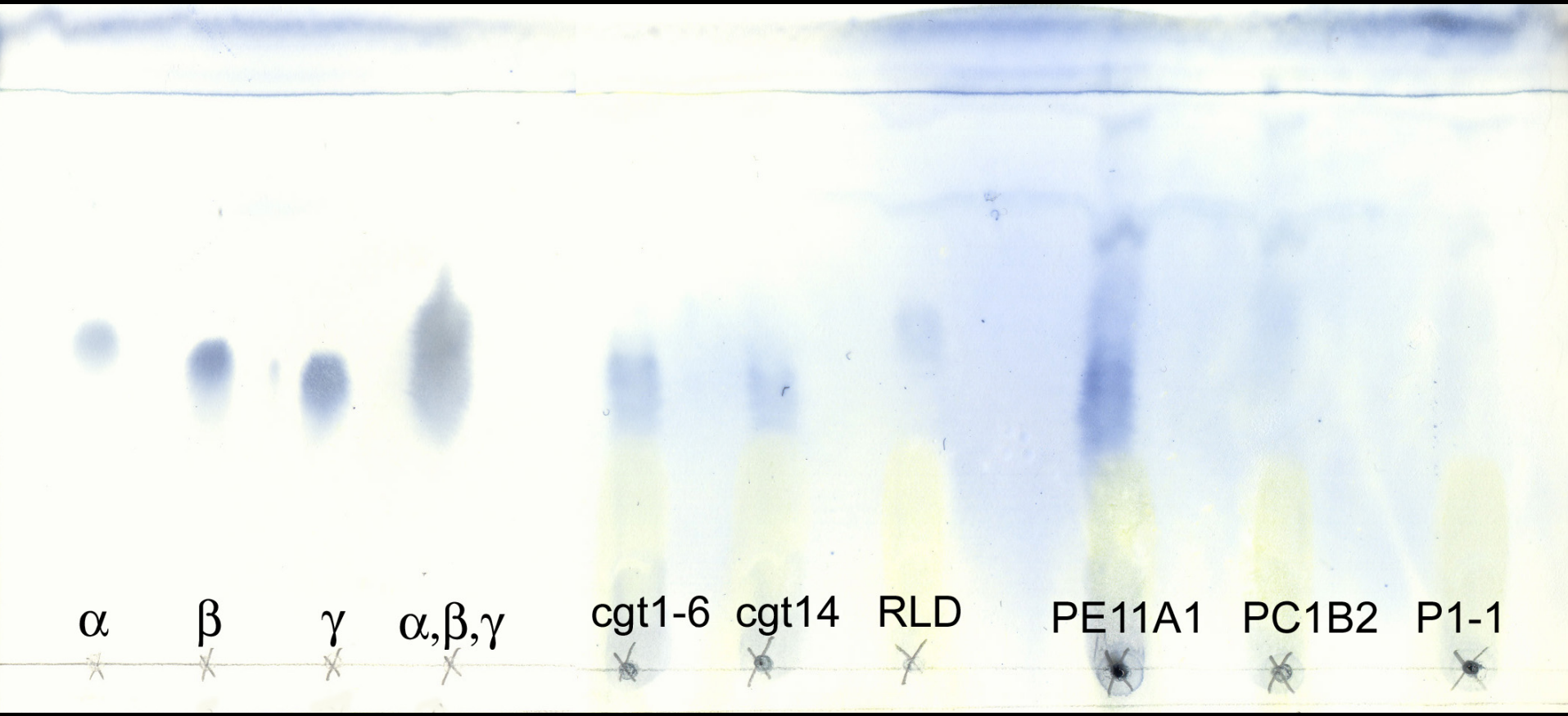
βCD Production in Arabidopsis Using the Phenolphthalein method



βCD Production in Tobacco



TLC of Cyclodextrins produced by plants



2μl spotted 4 times, mobile phase was acetonitrile-water-ammonium hydroxide (6:3:1)
Sprayed with Vaugh's solution
Developed using a hot plate.

Part III: PAH Phytoremediation

Soil was collected from the Ford Rouge Facility ~2000ppm tPAH

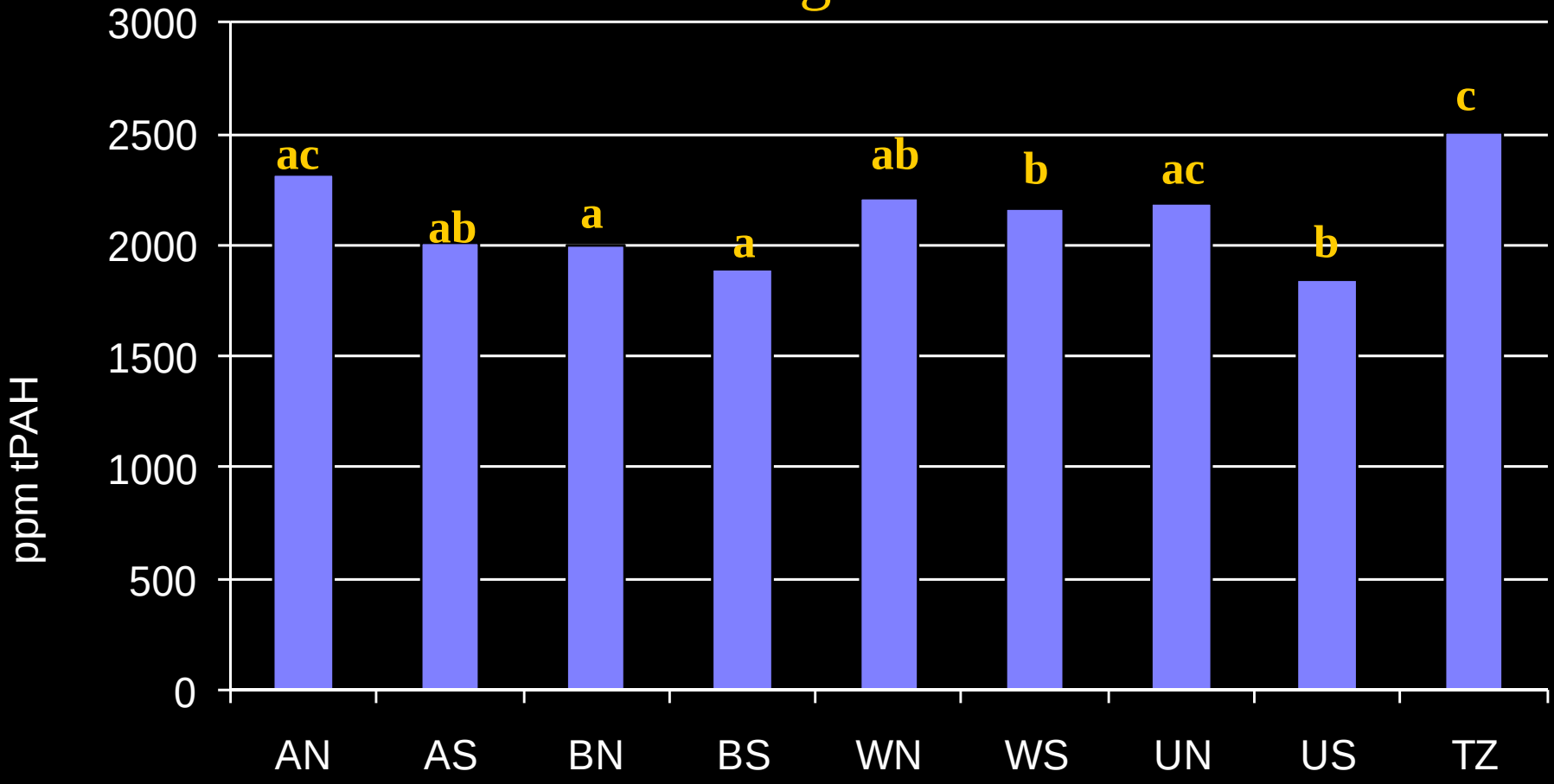
1. Soil added to Aluminum foil wrapped glass tubes

2. Planted with wild type and 2 lines of cgt-tobacco

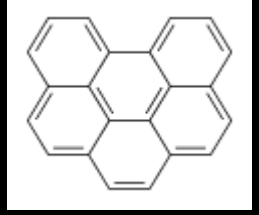
3. Half of plants were watered once with 2ml of 1% starch and were harvested 50 days after planting

Rouge PAH soil X cgt-Tobacco

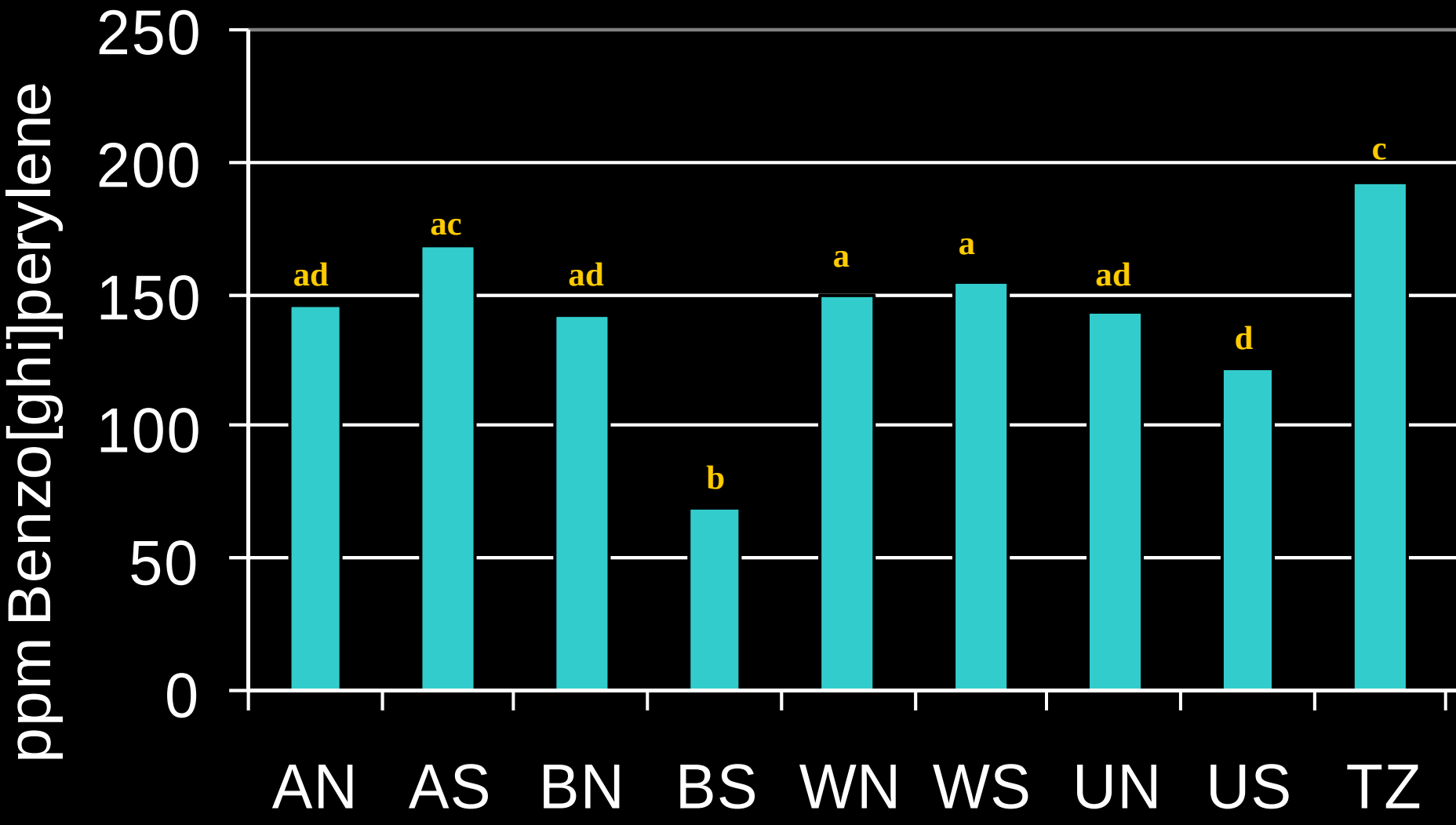
Starch addition and Transgenic plant effects on tPAH degradation



A = Cgt-Tobacco-PE13A2 B = Cgt-Tobacco-PC1B2 W = Wildtype
U = Unplanted N = No starch addition S = Starch added.



Starch addition and Transgenic plant effects on Benzo[ghi]perylene degradation



A = Cgt-Tobacco-PE13A2 B = Cgt-Tobacco-PC1B2 W = Wildtype
U = Unplanted N = No starch addition S = Starch added.

Conclusions

1. PI-cgt is a true novel CGTase grouping with other known Bacillus CGTases

2. PI-cgt is expressed and secreted in both plants and bacteria.

3. Plants expressing PI-cgt can have a positive effect on phytoremediation of some PAHs

4. Cgt-plants can be part of an integrated remediation system, especially with improvements in expression

Thank You!