Metabolic Engineering

Plant Metabolic Engineering for Pharmaceutical Production

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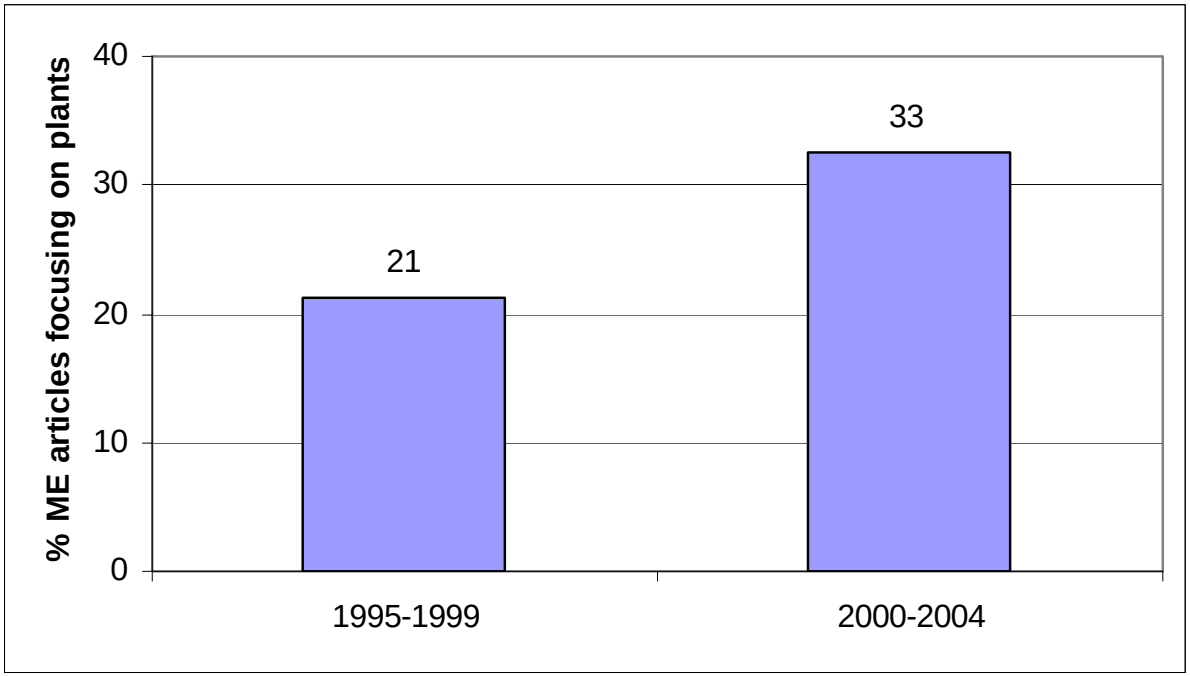
Emerging emphasis in plant ME

- New Gordon Conference in "Plant Metabolic Engineering"; July 10-15, 2005, Tilton, NH
  - » "This conference will provide a setting for developing multidisciplinary collaborations needed to unravel the dynamic complexity of plant metabolic networks and advance basic and applied research in plant metabolic engineering."



Web of Science; publications containing "metabolic engineering" and "plant(s)"

Key focus areas

- Nutritional value of foods (essential amino acids, oil seed crops, beta-carotene)

"Golden Rice" - enriched w/ vitamin A

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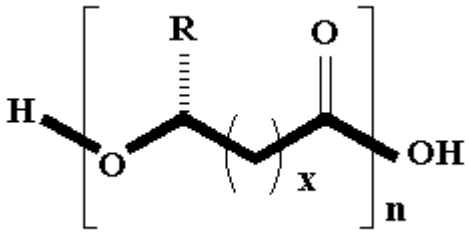
where nature performs™



- Polymer production (polyhydroxyalkanoates)

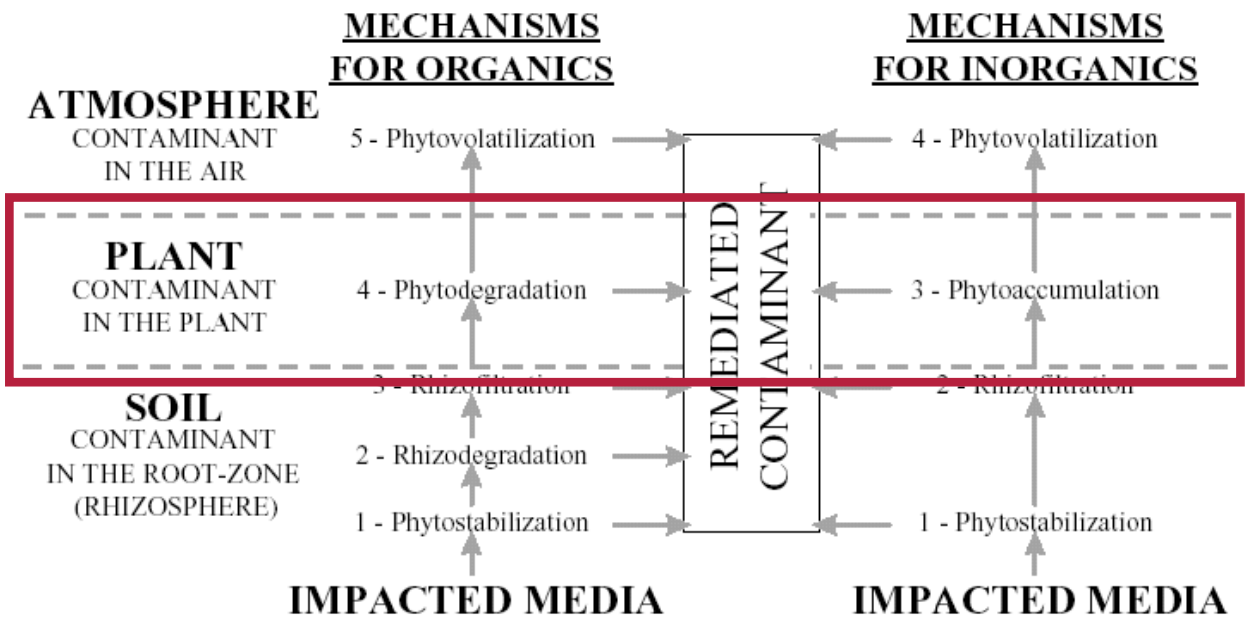
- Enhanced crop function for insect resistance, disease, drought, enhanced fertility, growth, nitrogen assimilation

MONSANTO
imagine™

Roundup Ready® canola, soybeans, corn, cotton

Key focus areas (cont.)

- Mathematical modeling (lipid synthesis, primary metabolism, secondary metabolism)
- Development of experimental tools (inducible promoters, proteomics, metabolite profiling)
- Phytoremediation (heavy metal uptake, e.g., arsenic, selenium, cadmium)



- **<u>Pharmaceutical production (indole alkaloids, taxanes)</u>**

Challenges for plant ME

- Compartmentalization (both within the cell and within the plant); localization issues complicate metabolite and flux analyses; redundancy of pathways
- Current models lack predictive quality
- Dearth of inducible promoters; critical for organ/cell specific expression
- Transformation of plants with multiple genes
- Lack of genetic characterization - 2 sequenced plant genomes (*Arabidopsis* and rice) as compared to 220 bacterial/archaebacterial, 11 fungal and 11 animal; often pathway enzymes are not cloned

Bioprocesses for pharmaceutical supply

- Common for the production of valuable products by microorganisms
  - » RecombinvaxHB® hepatitis B vaccine produced in recombinant *S. cerevisiae* (Merck)
  - » INTRON® Interferon alfa-2b - treatment for hepatitis and various cancers - produced by recombinant *E. coli* (Biogen)
- Mammalian cell culture processes
  - » EPOGEN® Erythropoietin - glycoprotein used to treat anemia associated with chronic renal failure - produced by recombinant Chinese Hamster Ovary (CHO) (Amgen)
- Plant processes are now emerging due to advances in metabolic engineering technologies

Plant cell and tissue culture
"Best of both worlds"

- Advantages over other cell culture/fermentation systems
  - » safety: low risk of contamination by mammalian viruses, pathogens, toxins
  - » capability for advanced post-translational processing
- Advantages over agriculture "whole plant"
  - » suitable when small-to-medium quantities of high-value, high-price products are needed
  - » time involved for production is significantly less than necessary for agriculture
  - » regulatory requirements may be met more easily due to highly controlled process conditions, e.g., lack of variability in cultivation conditions
  - » product recovery and purification simpler when product is secreted to extracellular medium
- Potential products
  - » animal-derived proteins
  - » vaccines
  - » **plant-derived products**

Valuable plant-derived products

Flavors, Colors, Agricultural Chemicals, Pharmaceuticals

- ajmalicine, serpentine (anti-hypertensive)
- camptothecin (anti-HIV)
- hypericum (anti-rheumatism)
- sanguinarine (anti-microbial)
- **Paclitaxel**, vinblastine, vincristine (anti-cancer)

Hypericum perforatum

Catharanthus roseus

Taxus baccata

Paclitaxel

- Paclitaxel (Taxol®) is a leading anti-cancer agent

Approved by the FDA for the treatment of breast, ovarian, lung cancers as well as Kaposi's sarcoma

Numerous current clinical trials

» test the effectiveness of paclitaxel against leukemia, lymphoma, as well as cancers of the lung, head and neck, and colon

» the application of paclitaxel earlier in the course of disease

» combinatorial treatments with other anti-cancer drugs

- Produced successfully in **plant** cell cultures (*Taxus*)
- Supply is limited through natural resources (yew tree)
- Plant cell culture technology is being utilized to meet supply demands (major success story!)

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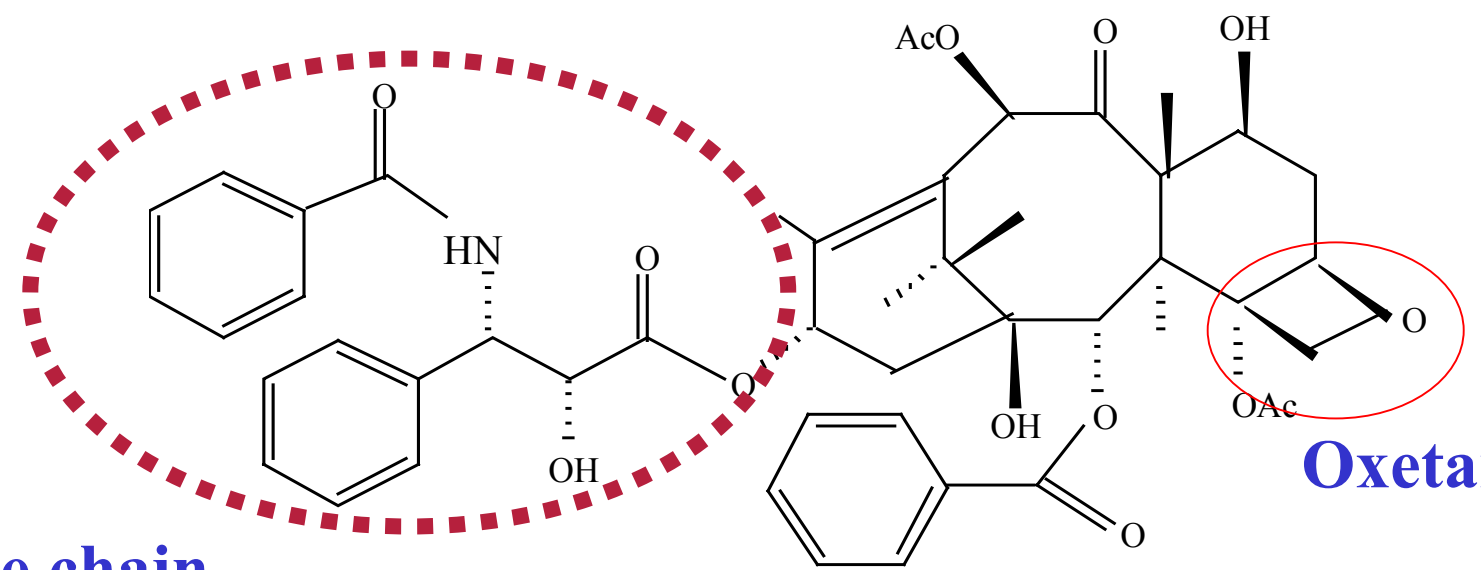
Paclitaxel structure

- Paclitaxel is a member of a family of "taxanes"
  - » secondary metabolite
  - » characteristic oxetane ring and side chain are necessary for high level of anti-cancer activity
  - » taxanes can be classified as side-chain or nonside-chain



Production and supply of plant products

- Harvestation
  - » often limited natural resources due to slow growth of source plant
  - » difficult product recovery and low yield
- Semi-Synthesis
  - » economical if precursor is available
  - » precursor availability and reliability
- Chemical Synthesis
  - » low product yields
  - » very expensive
- Bacteria/Yeast
  - » complex pathways not easily reconstituted
  - » extremely low yields
- **Plant Cell and Tissue Culture**

Plant growth models for culture and ME

- Organ cultures
  - » Shooty teratomas, hairy roots
  - » Advantages: differentiated; higher production of secondary metabolites, ease of transformation, increased genetic stability
  - » Disadvantages: scale-up difficult due to oxygen and nutrient limitations

J. Shanks, Iowa State
C. roseus

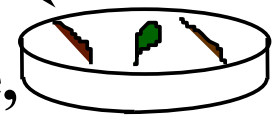
- Suspension cultures
  - » Advantages: ease of scale-up using traditional methods, compliance with GMP procedures
  - » Disadvantages: long-term stability in productivity

T. cuspidata

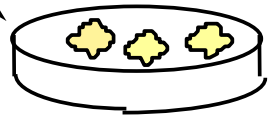
Plant Cell Culture

plant or embryo

Expose live tissue, sterilize, and plate on solid medium

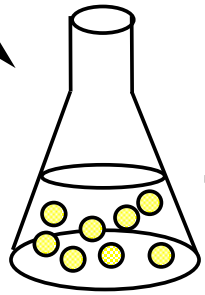


callus formation



Screen for product of interest

put callus into liquid medium



formation of suspension culture cell lines

Advantages of plant cell culture

- Not reliant on field grown materials/year-round production
- Cleaner/simpler product recovery
- Production of novel compounds not found in the natural plant
- Controlled process with easy manipulation for optimization of production conditions
- Can be easily applied to study cellular metabolism where results are applicable to whole plant systems
- Cells can be metabolically engineered for desired properties

Difficulties with plant cell culture

Characteristic	*Microorganism*	*Plant Cell*
Size	2 μm	20 μm +
Shear Stress	Insensitive	Sensitive
Doubling Time	< 1 hour	Days
Fermentation Time	Days	Weeks
Product Accumulation	Medium	Often cell-Associated
Production Phase	Uncoupled	Often growth-associated
Variability in accumulation	Low	High
Product yields	High	Low

Process development

Cell Line Selection and Development
(callus initiation from embryos to suspension culture)

↓

Medium Optimization

↓

Traditional Productivity Enhancement
(optimize temperature, immobilization, bioreactor design, etc.)

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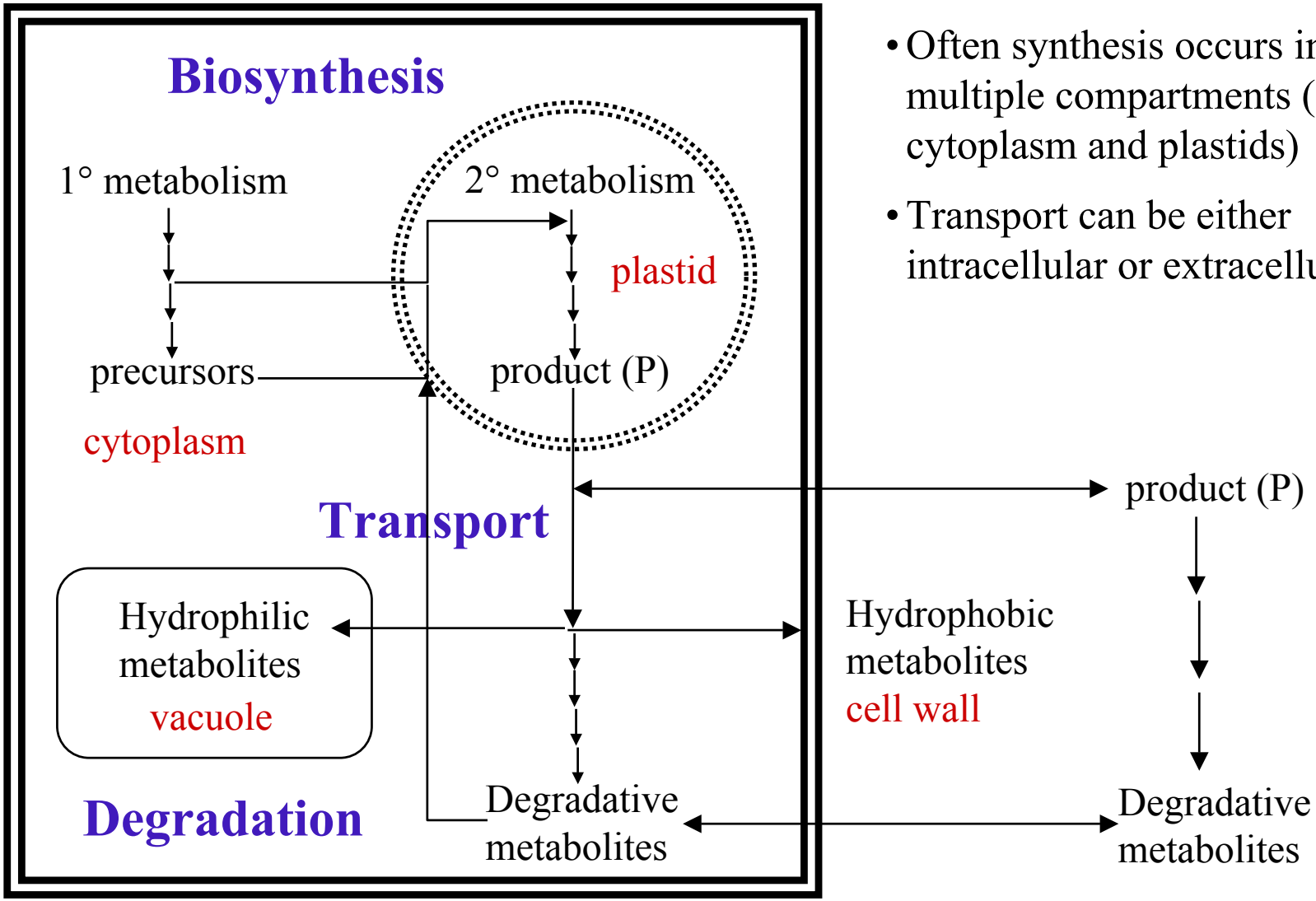
Understanding of Cellular Metabolism and Regulation so Directed Metabolic Engineering Strategies can be Implemented

Overview of cellular metabolism



- Often synthesis occurs in multiple compartments (e.g., cytoplasm and plastids)
- Transport can be either intracellular or extracellular

Targeted Metabolic Engineering for Enhanced Paclitaxel Accumulation in Plant Cell Culture

Funding: National Institutes of Health (Metabolic Engineering Program)

Collaborators: Dr. Elsbeth Walker, UMass Department of Biology, Dr. Donna Gibson, U.S. Plant Soil and Nutrition Laboratory (USDA)

Students: Zeke Nims, Nadia Boutaoui, Camille Dubois, Kham Vongpaseuth

Metabolic manipulation

Altering cellular metabolic pathways to achieve a desired state

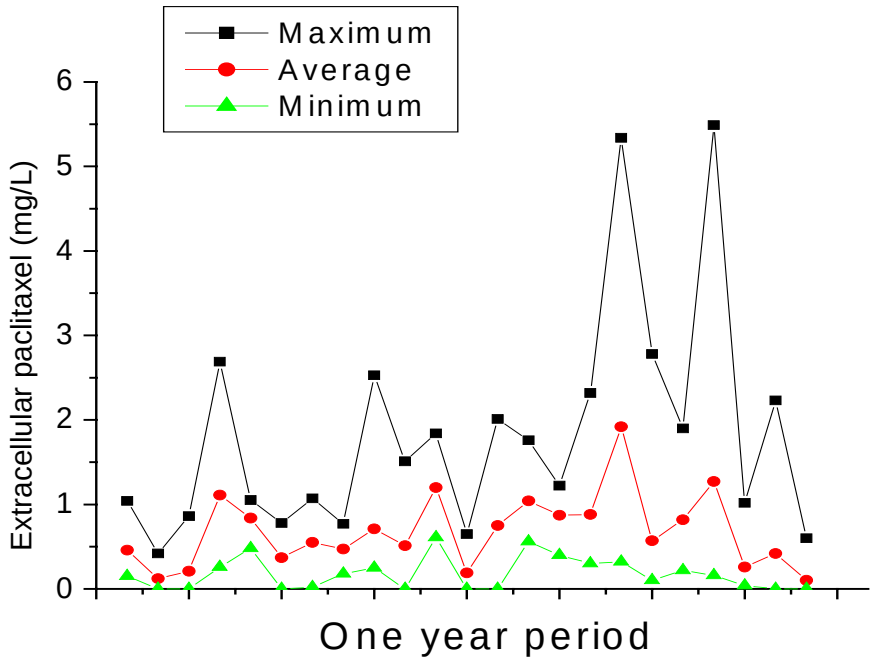
Approaches

- Enzyme elicitation - increasing the activity or pool of specific enzymes that catalyze desired biochemical reactions
- Metabolic inhibitors - inhibition of specific metabolic steps to characterize metabolism and/or achieve a desired result
- Precursor feeding - addition of specific metabolites to increase flux through a desired pathway
- Metabolic engineering - genetic transformation; alteration of specific enzymatic or transport pathways

Goal: Characterize paclitaxel metabolism and regulation to design targeted strategies for increased selectivity and yield

Challenges

- Paclitaxel accumulation is highly variable



- *Taxus* genome is not sequenced and therefore system-wide expression analysis is not possible
- Regulation may not be limited to biosynthesis; may involve transcriptional control, transport or degradation
- No stable transformation method developed for *Taxus*

Two-pronged approach

Traditional
Pathway-level

Using known pathway genetic information, identify bottlenecks and control genes

Novel
Systems-level

Apply new molecular techniques to identify and isolate previously unidentified control genes

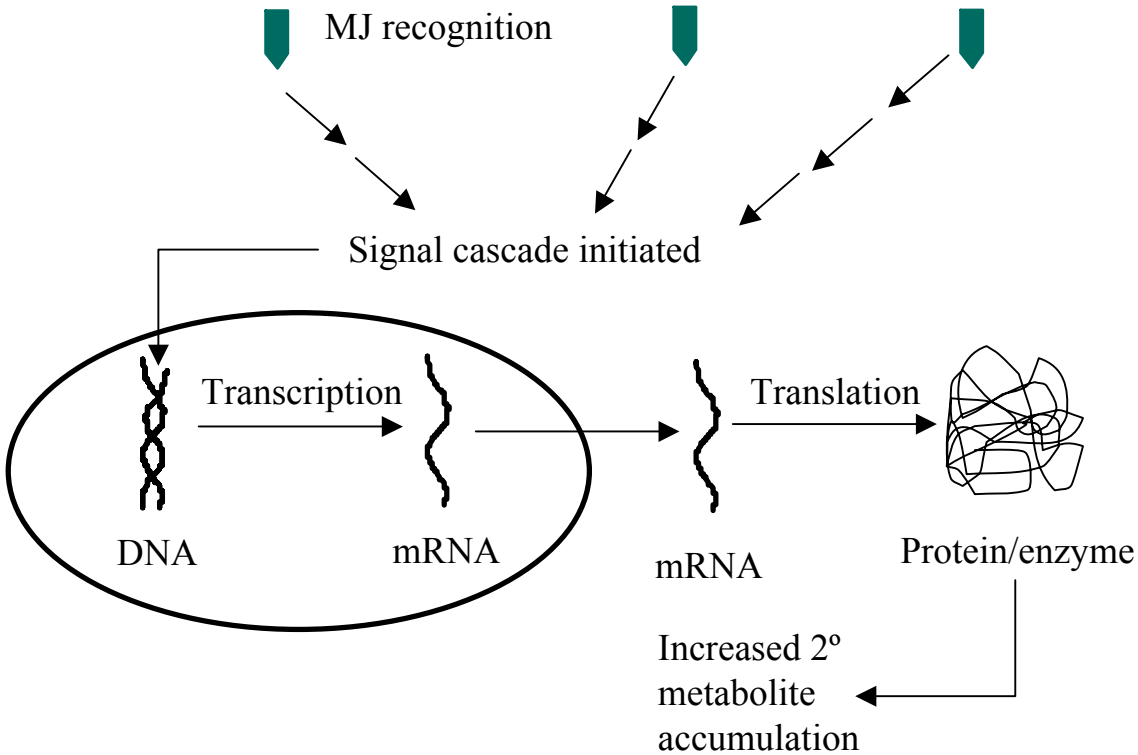
Targeted metabolic engineering strategies to increase paclitaxel yields

Enzyme elicitation

Elicitation is used to induce taxane accumulation and upregulate gene expression; unelicited cultures produce no/low levels of key taxanes

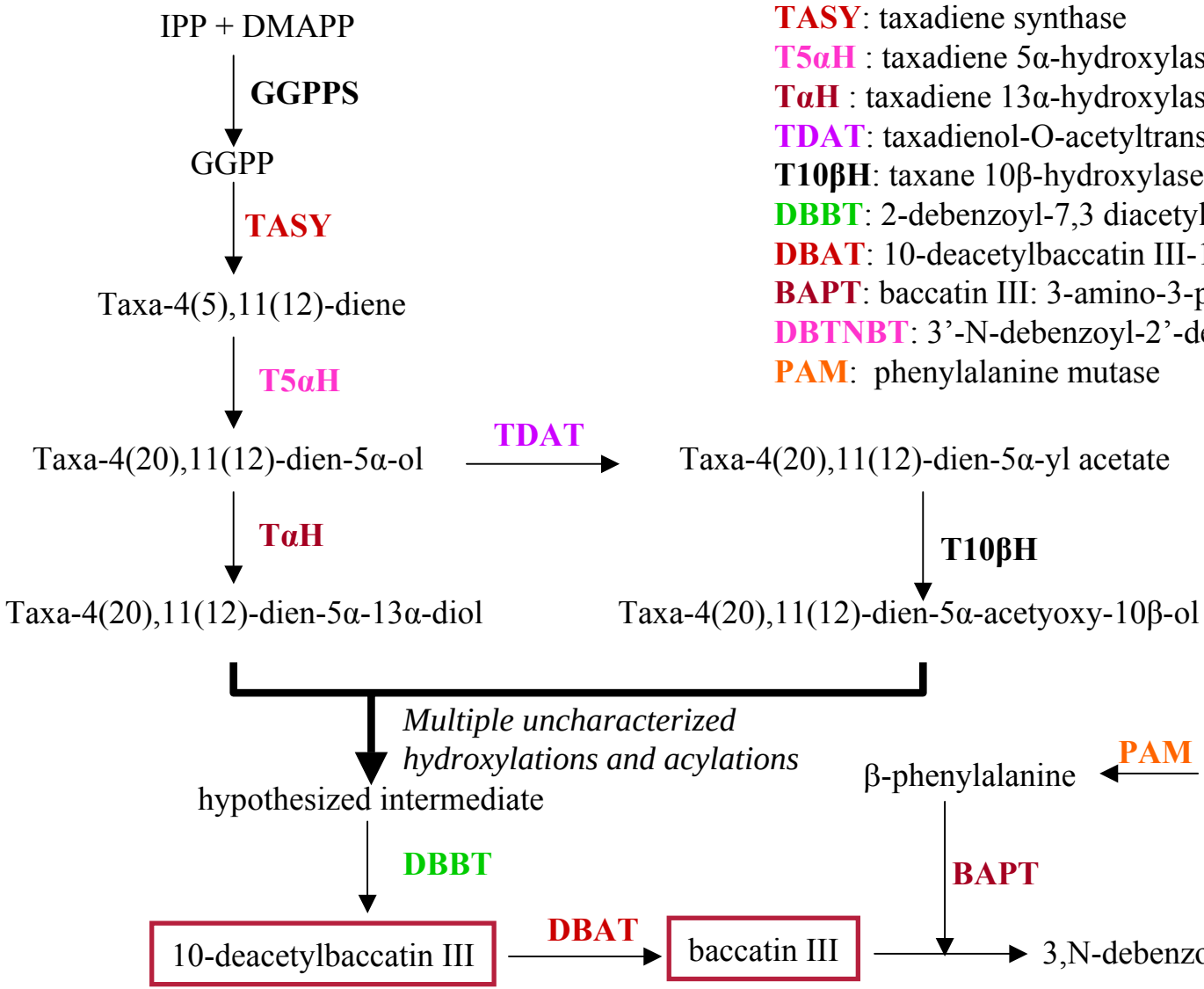
Methyl jasmonate (MJ)

- Synthesized via lipoxygenase pathway and produced in response to wounding
- Elicits secondary metabolite synthesis in a wide-range of plant cell culture systems
- Paclitaxel synthesis shown to increase substantially with MJ elicitation



Taxane biosynthesis

GGPPS: geranylgeranylpyrophosphate synthase
TASY: taxadiene synthase
T5αH : taxadiene 5α-hydroxylase
TαH : taxadiene 13α-hydroxylase
TDAT: taxadienol-O-acetyltransferase
T10βH: taxane 10β-hydroxylase
DBBT: 2-debenzoyl-7,3 diacetyl baccatinIII-2-O-benzoyltransferase
DBAT: 10-deacetylbaccatin III-10-o-acetyltransferase
BAPT: baccatin III: 3-amino-3-phenylpropanoyltransferase
DBTNBT: 3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase
PAM: phenylalanine mutase



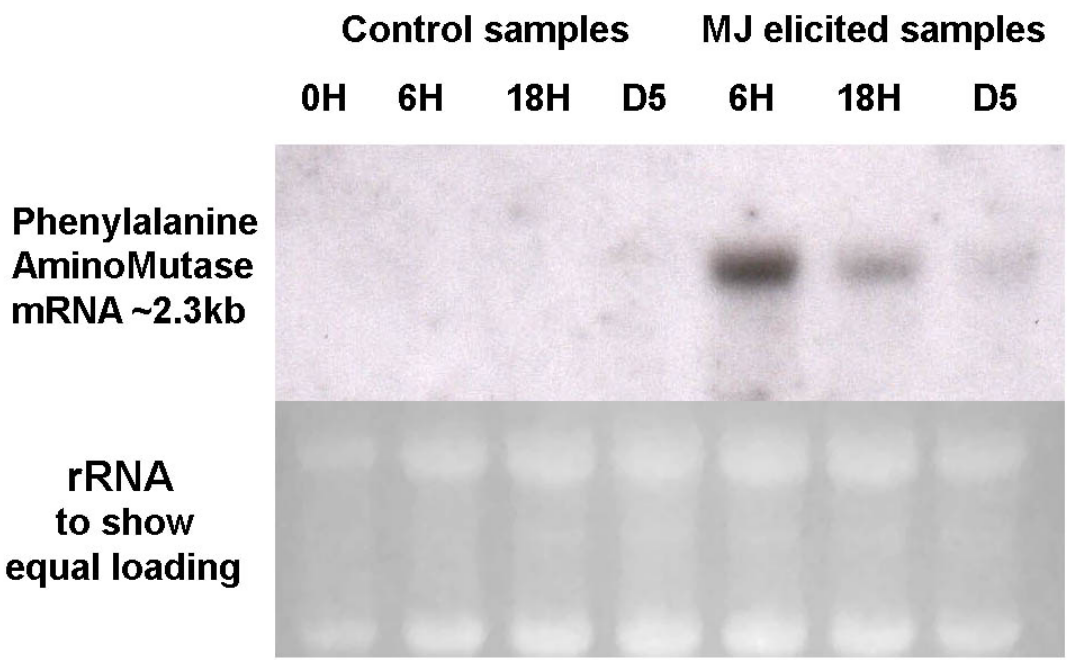
I.

<u>Traditional</u>
Pathway-level

Using known pathway genetic information, identify bottlenecks and control genes

Transcript profiling

- Primers designed using conserved regions of previously cloned genes from *T. cuspidata*, *T. brevifolia*, and *T. canadensis* (available from GenBank)
- Probes generated via PCR for Northern blot analysis
- Fragments labeled with ^{32}P nucleotides



100 μM MJ added on day 7 (control w/o MJ elicitation)

↓

Cells harvested at various time points (up to 7 days)

↓

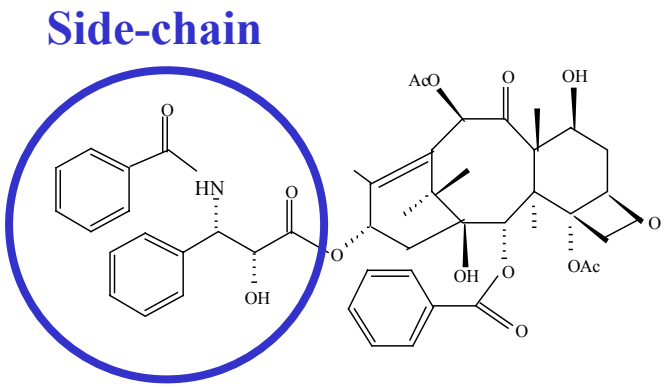
Total RNA prepared for transcript analysis

↓

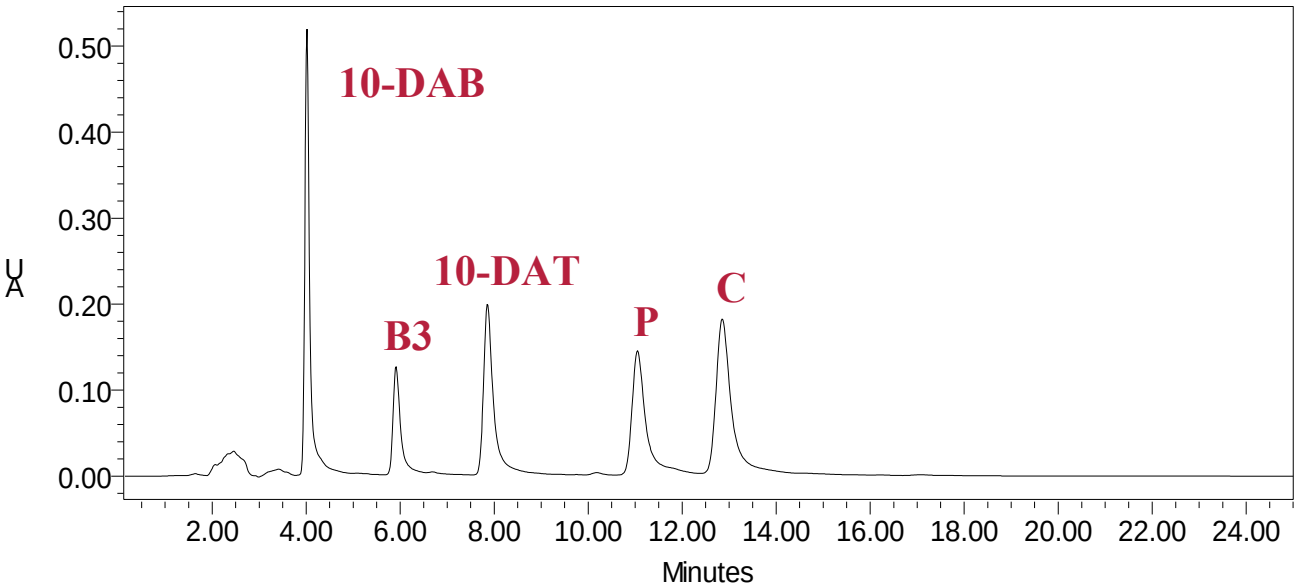
RNA transcripts separated and identified via radio-labeled probe hybridization

Metabolite profiling

- Nonside-chain taxanes
  - » baccatin III
  - » 10-deacetylbaccatin III
- Side-chain taxanes
  - » paclitaxel
  - » cephalomannine



Paclitaxel



100 µM MJ added on day 7 (control w/o MJ-elicitation)

↓

Cells harvested at various time points (up to 7 days)

↓

Samples prepared for **HPLC** analysis

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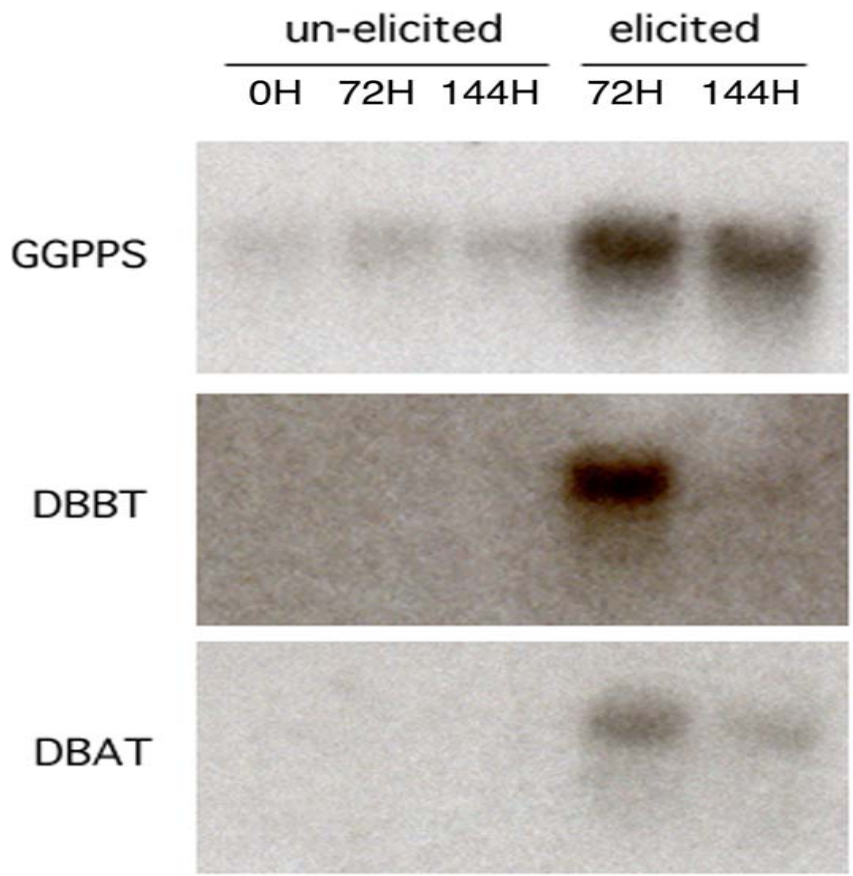
Peak identification through retention time and PDA spectral comparison with known standards; metabolites confirmed with **MS**

Effect of MJ on gene expression

Expression profiling of three representative pathway genes

- Upregulation of pathway genes following MJ elicitation
- Pathway genes differ in levels of constitutive expression
- Transcription level control is likely important for regulation of taxane biosynthetic pathway
- Collect expression data for all known pathway genes



P991 (*T. cuspidata*) cell line
Elicited on day 7 with 100 μM MJ

Summary - expression analysis

- Northern blot analysis, RT-PCR and metabolite profiling were used to probe taxane biosynthetic pathway regulation
- MJ elicitation induces taxane biosynthesis and gene expression
  - » Different physiological states were compared to determine potential rate-influencing steps in taxane biosynthesis
- Upstream taxane biosynthetic steps are highly upregulated by MJ elicitation
- Downstream steps are not upregulated with MJ elicitation

II.

<u>Novel</u>
Systems-level

Apply new molecular techniques to identify and isolate previously unidentified control genes

<u>Focus of ongoing work</u>

Approach

Use MJ to upregulate paclitaxel accumulation

Examine two physiological states (-MJ and +MJ)

↓

Identify genes that are consistently up- or down-regulated

Identify differentially expressed transcripts

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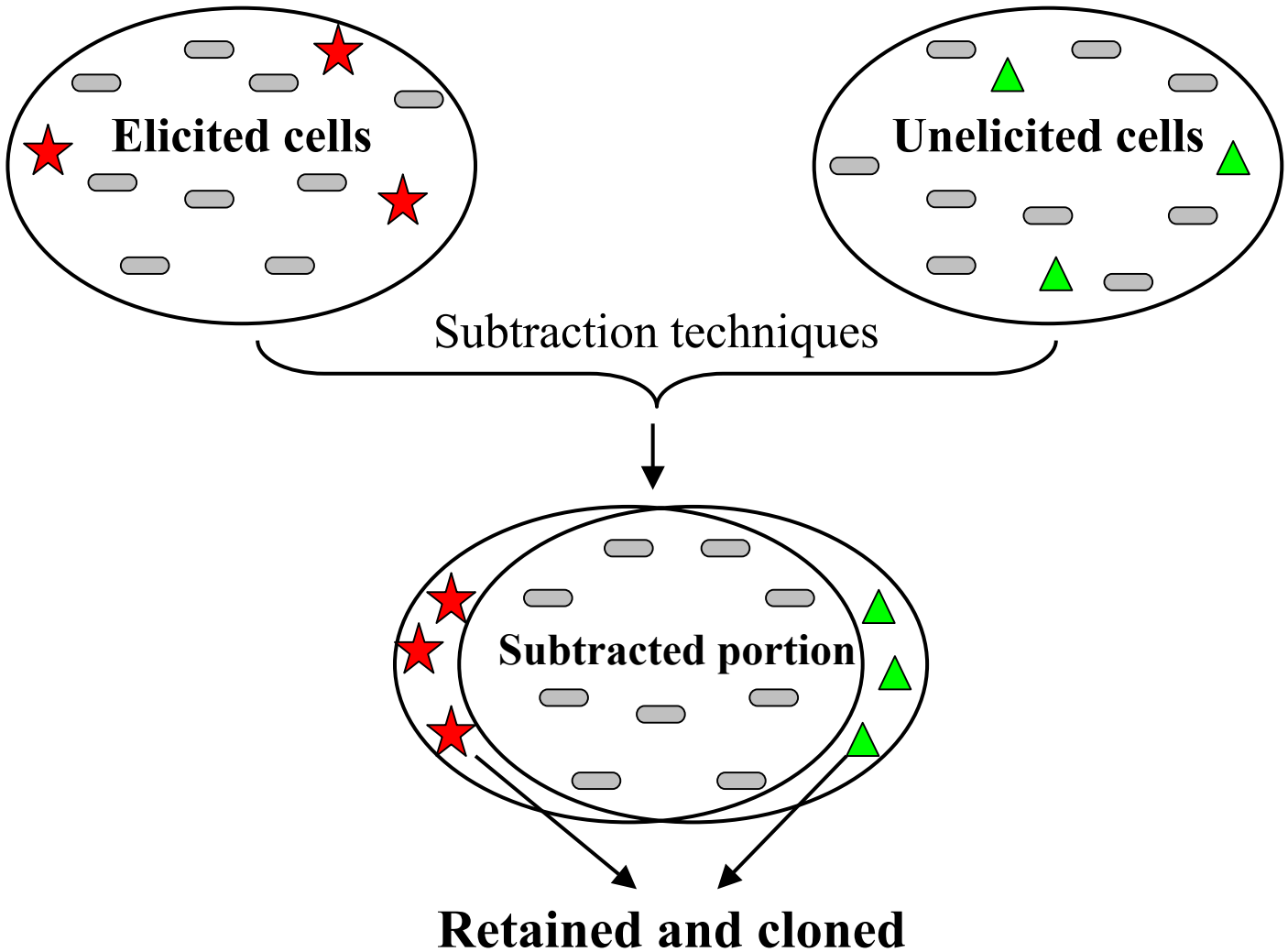
Predict gene function

Construct cDNA libraries
Sequence full-length control genes
Bioinformatics resources to predict gene function

↓

Transform *Taxus* cell lines and evaluate performance

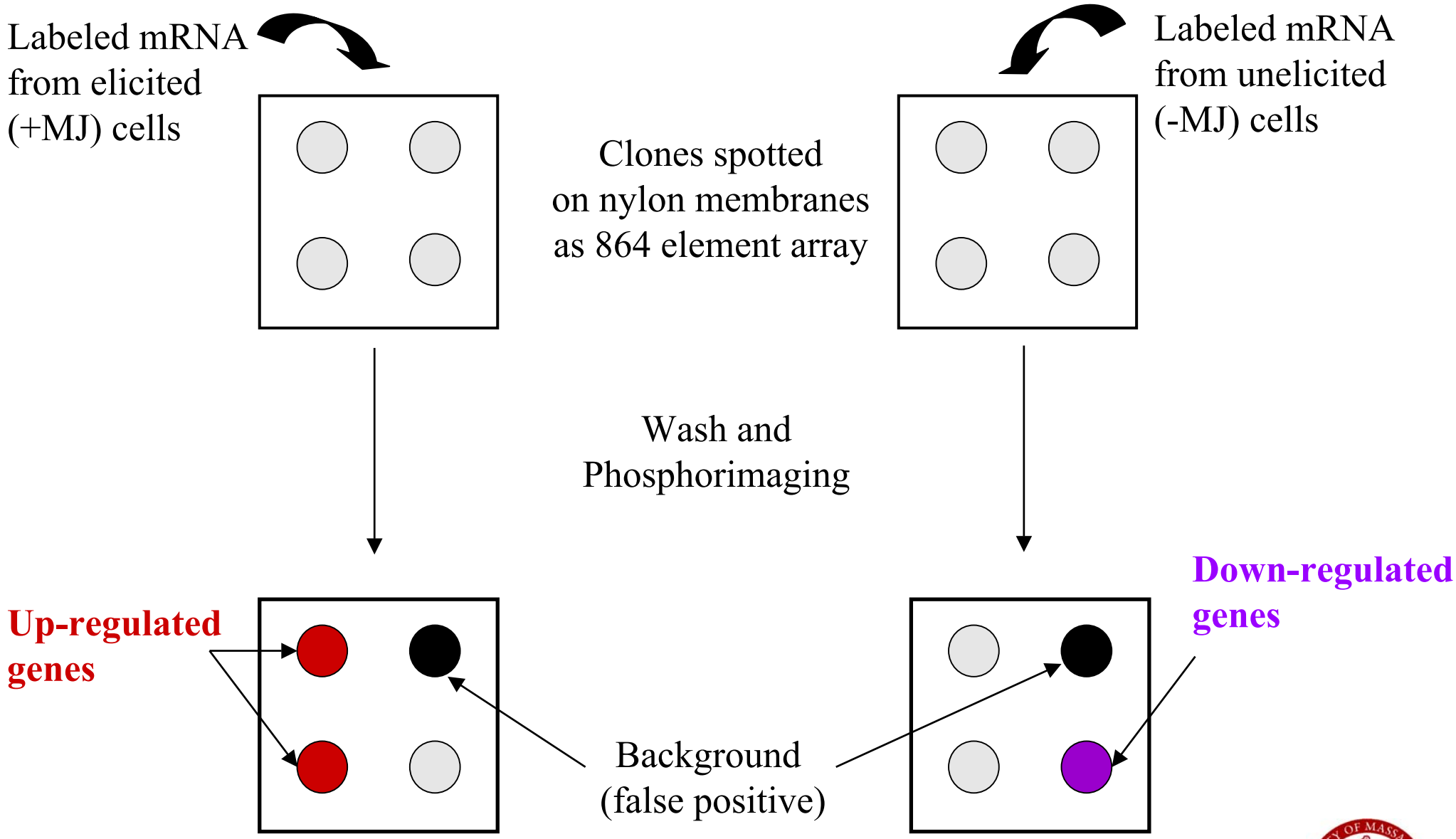
Differential gene expression



Problem: Presence of background from non-differentially expressed genes

Solution: Macroarray analysis can be used to screen large number of potentially relevant clones

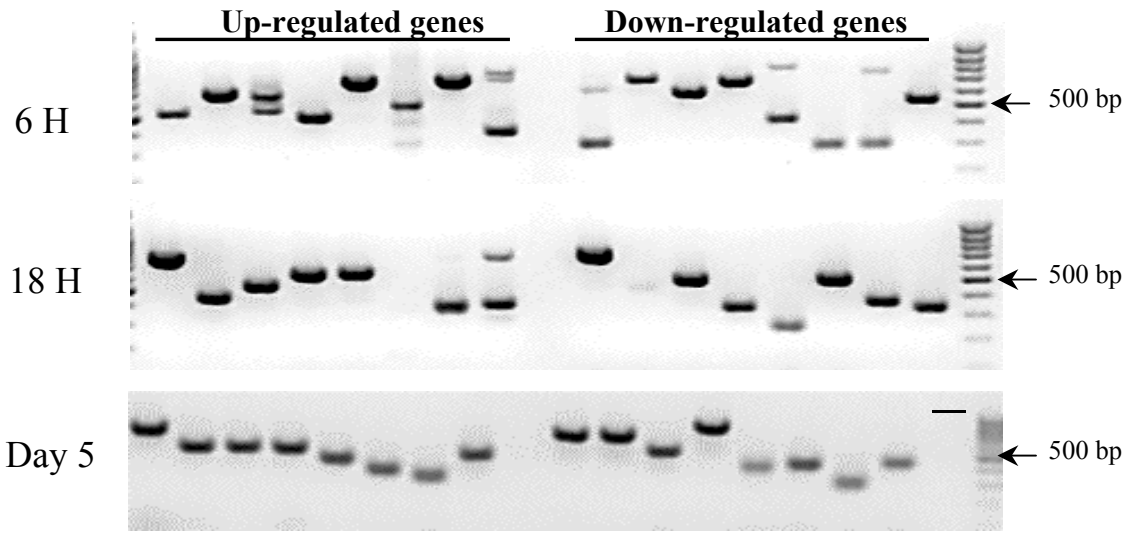
cDNA macro-arrays



Isolation of differentially expressed clones

Sample collection time (post MJ elicitation)	Targeted control genes	Number of clones from Forward subtraction (up-regulated genes)	Number of clones from Reverse subtraction (down-regulated genes)
6 H	MJ signaling	96	96
18 H	Paclitaxel accumulation	96	96
Day 5	Transport and degradation	96	96

Fragments of 8 clones picked at random from each SSH library



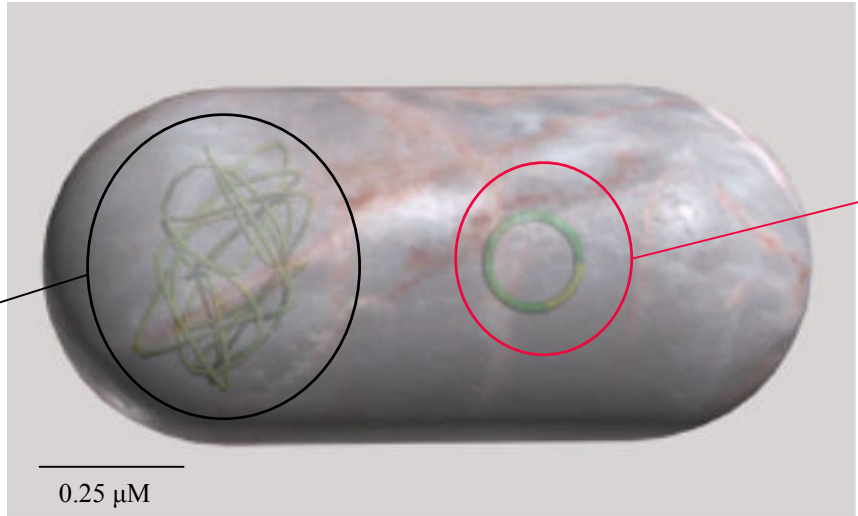
- There is significant variability of band sizes
- 32 different band categories (based on size) out of 48 clones - different potential control genes

Ongoing work

- Sequencing of ~600 clones from subtraction technique
- Construction of macro-arrays using ~600 clones
- Construction of cDNA library to isolate full-length control genes
- Establishment of a protocol for *Taxus* suspension culture transformation
- Transformation of late pathway genes that have been shown to exhibit limited upregulation upon MJ elicitation

Agrobacterium

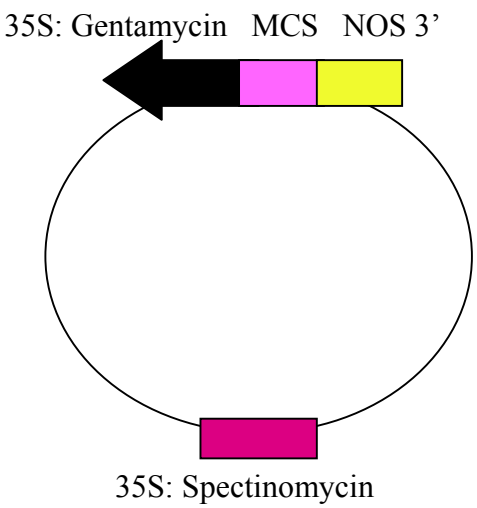
- Once key genes are identified, they may be transformed into *Taxus* cell cultures using *Agrobacterium tumefaciens*
  - » *A. tumefaciens* is a common soil bacterium that causes crown gall formation in plants by transferring some of its DNA to the host



- *A. tumefaciens* can be transformed with a secondary plasmid containing the gene of interest

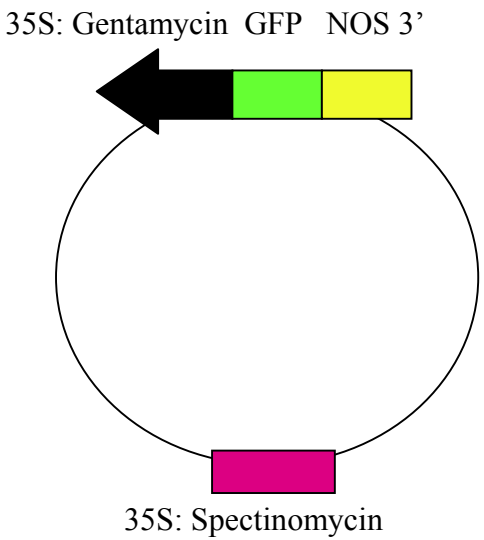
Transformation of *Agrobacterium*

PZP221



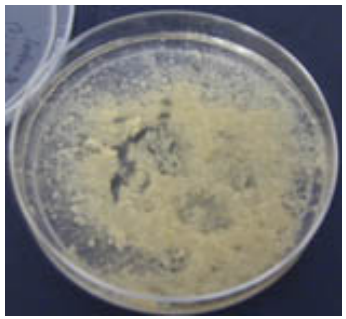
"negative control"

35S:GFP



"model gene - GFP"

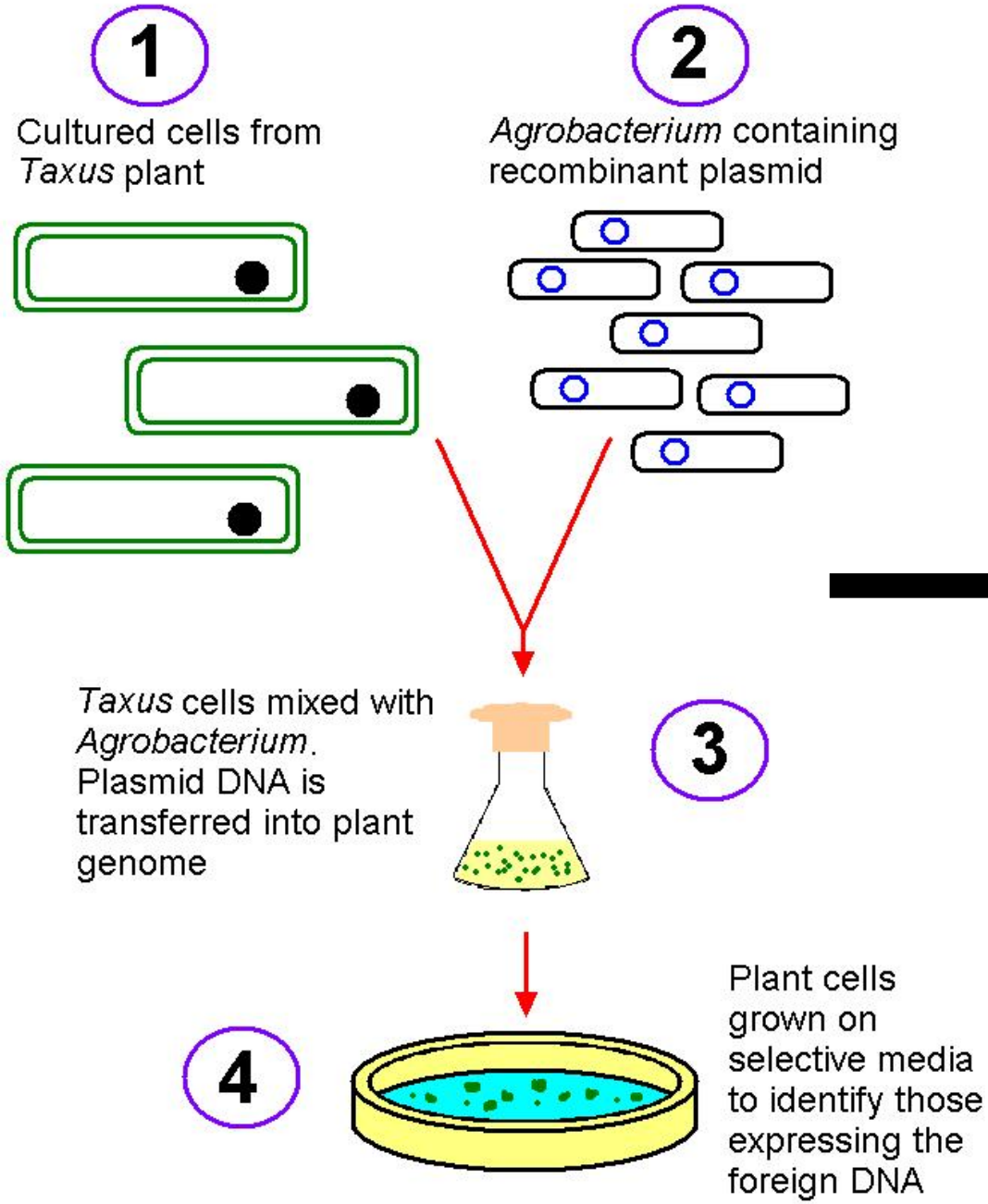
- Spectinomycin resistance allows for selection of transformed *Agrobacterium*
- Gentamycin resistance allows for selection of transformed *Taxus* cells



- Gentamycin

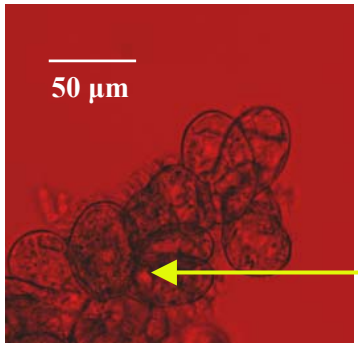
+ Gentamycin

Agrobacterium-mediated transformation



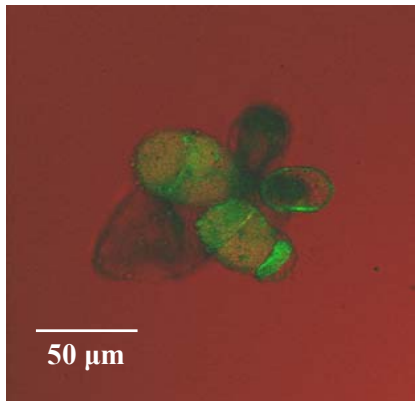
Transfer callus to suspension to generate engineered cell lines for characterization and use in bioprocesses

Transformation results



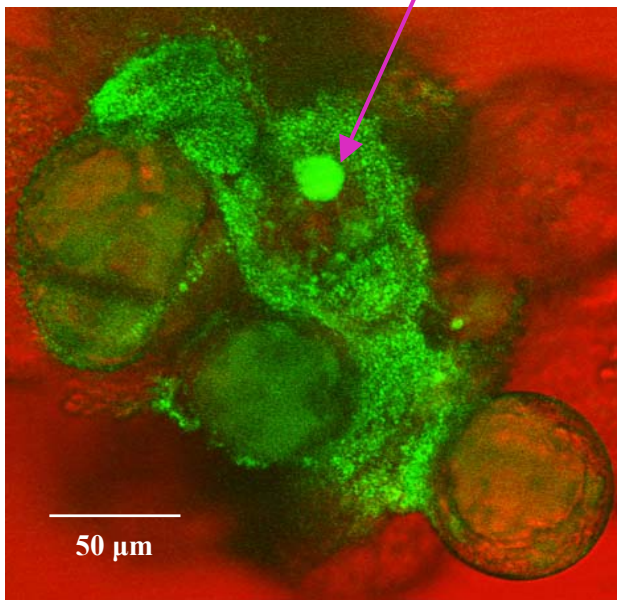
(A)

Aggregate of live PO93X showing no GFP fluorescence in the nuclei (A). Illustrates appearance of *Taxus* cells before transformation.



PO93X cells transformed with the PZP221vector (negative control) showing autofluorescence of the cell wall. Autofluorescence may be an effect of the co-cultivation with *Agrobacterium*.

(B)



PO93X cell showing a nuclear-localized GFP signal (B), along with autofluorescence of the cell wall.

Summary: results-to-date

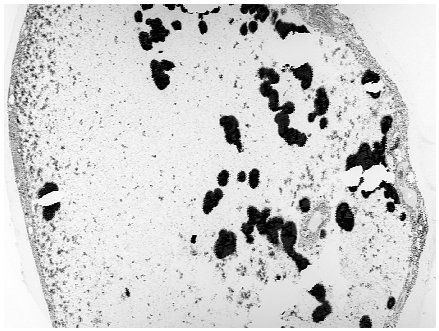
- Paclitaxel rate-influencing pathway genes have been identified
- 600 differentially expressed clones in response to increased paclitaxel accumulation have been isolated - these clones represent potential *global metabolic control genes*
- Transformation of *Taxus* suspensions has been accomplished and is currently being optimized

Impact

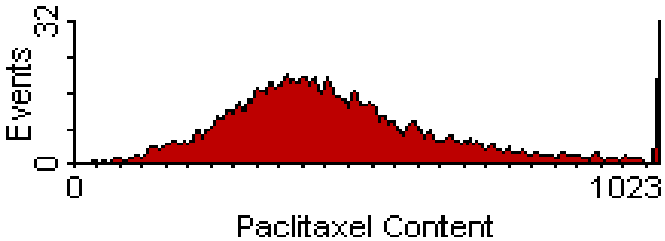
- New approach for targeted application of metabolic engineering to plant systems with unsequenced genomes and minimal genetic characterization
- Improved processes for production of plant pharmaceuticals from cell culture
- Transformation technology for *Taxus* suspensions and generation of transgenic cell lines for use in bioprocesses

Where do we go from here for plant ME?

- Cell culture heterogeneity; population analysis
- Emphasize industrial important organisms; bring focus off "model" system
- New approaches for the study of genetically uncharacterized plant systems
- Transform multiple genes for desired outcome



Day Post Transfer	Paclitaxel (mg/L)	% of cells "specialized"
2	0.0	0%
8	2.5	5%
15	10	10%



- Metabolic engineering for suppression of undesired pathways
- Systems approaches; focus not only on biosynthetic pathways; important in process design

Acknowledgments

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- N. Ezekiel Nims (Ph.D.)
- Nadia Boutaoui (Post-doc
- David Babson (B.S.)

Collaborators

- Prof. Elsbeth Walker
  Biology
- Prof. Jennifer Normanly
  Biochemistry and Molecular Biology
- Dr. Donna Gibson (USDA)
- Prof. Michael Shuler (Cornell)

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