Making vanillin the natural way- how plants do it

Richard A. Dixon
BioDiscovery Institute and Department of Biological Sciences,
University of North Texas

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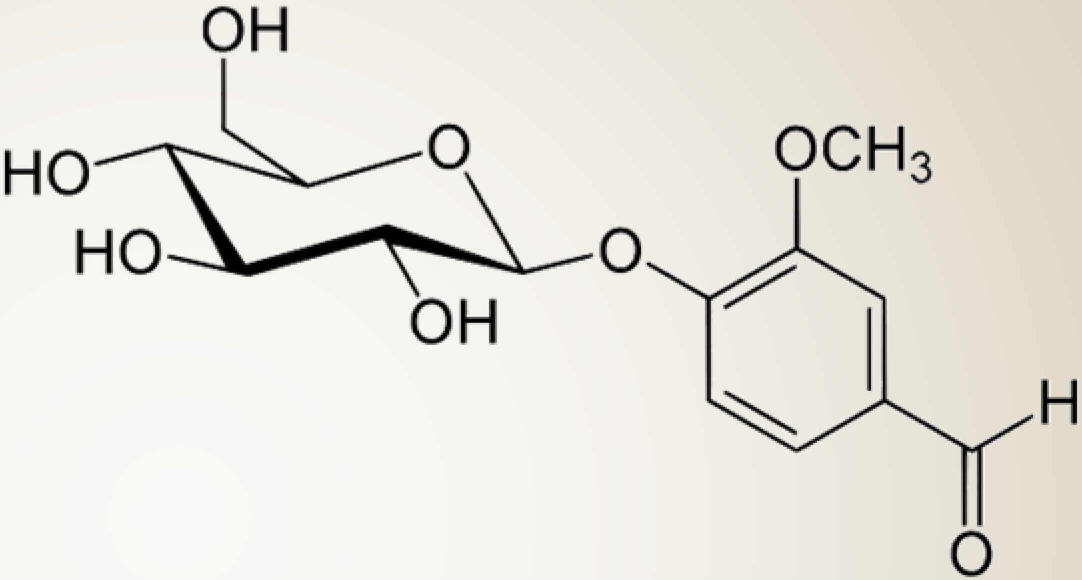
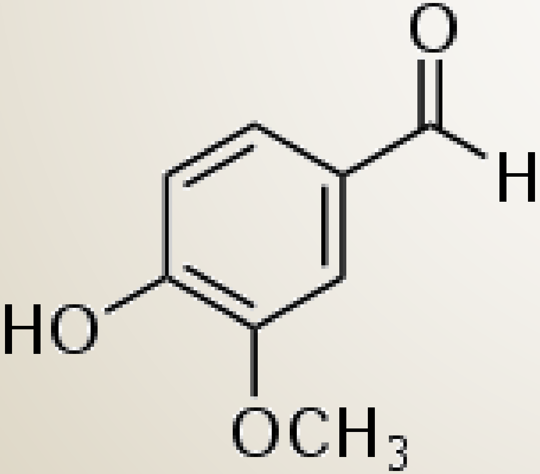
Vanillin is the principal component of vanilla flavor





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Ways to increase “biological” vanillin production

- Improve the yield of vanilla pods
- Increase the biotic and abiotic stress resistance of the vanilla orchid
- Increase the yield of vanillin in *V. planifolia*
  - Increased flow through the natural pathway
  - Diversion of metabolites into the vanillin pathway
- Introduce the vanillin pathway into other plants
- Process engineer plant biomass to vanillin
  - Chemical
  - Biological

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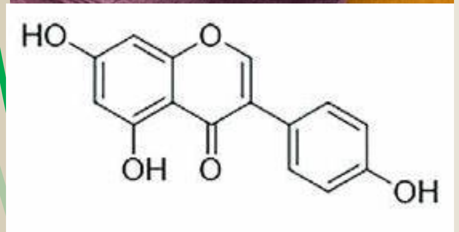
Engineering metabolic pathways in plants is not that difficult

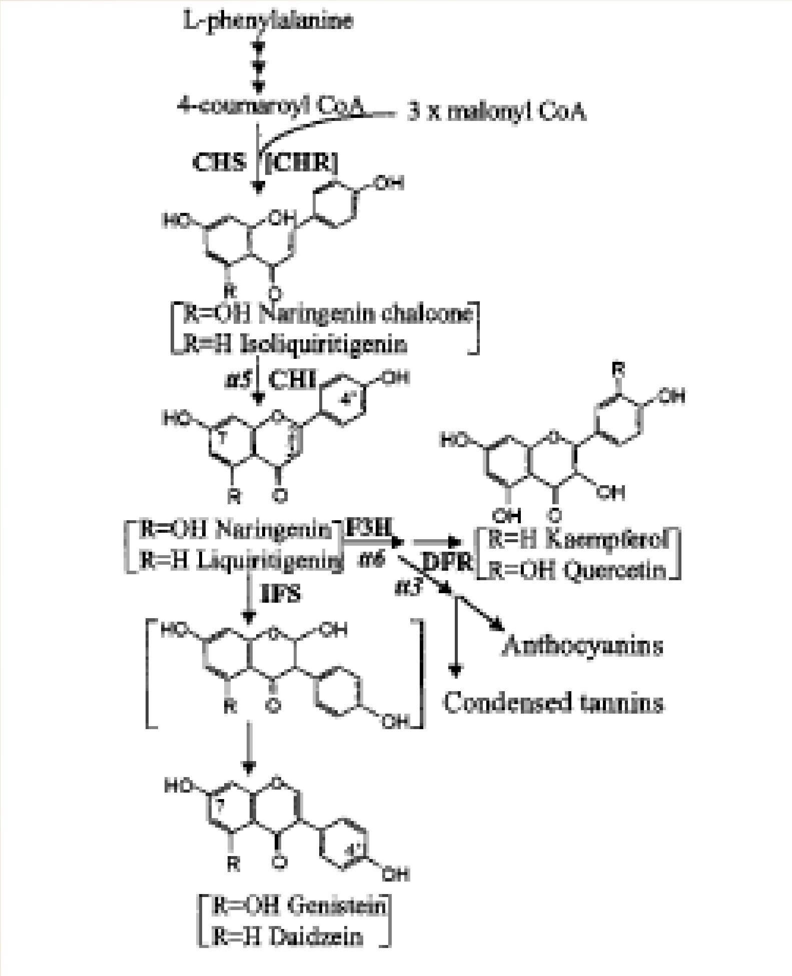
Bottlenecks for metabolic engineering of isoflavone glycoconjugates in Arabidopsis

Chang-Jun Liu, Jack W. Blount, Christopher L. Steele*, and Richard A. Dixon†

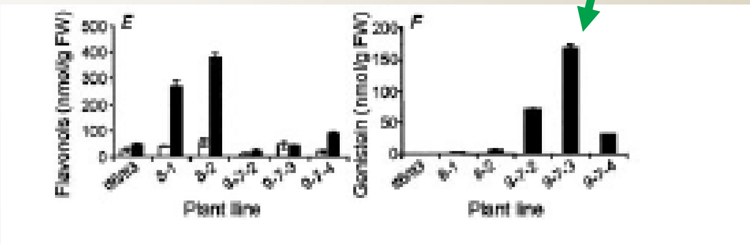
14578 –14583 *I PNAS I* ***October 29, 2002*** *I vol. 99 I no. 22*



Genistein (phytoestrogen)



Genistein



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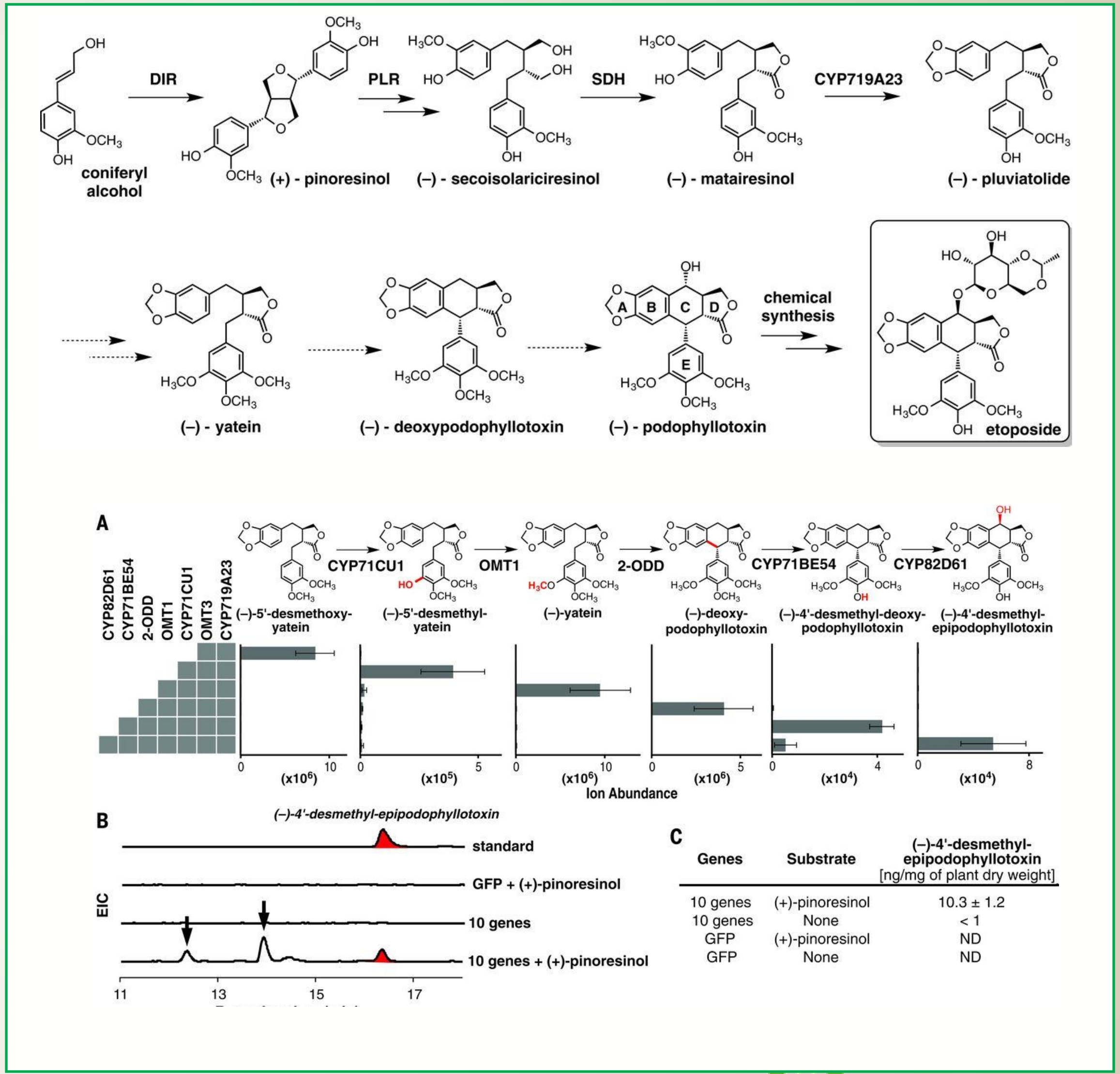
….and way more complicated pathways can now be engineered

Six enzymes from mayapple that complete the biosynthetic pathway to the etoposide aglycone

WARREN LAU AND ELIZABETH S. SATTELY

SCIENCE 11 Sep 2015 Vol 349, Issue 6253 pp. 1224-1228



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So it should be easy to engineer vanillin in plants, right?

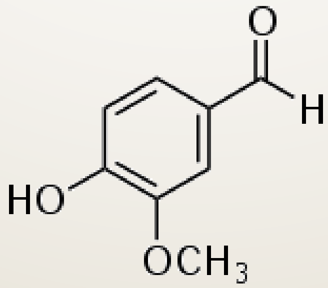
What do we need?

A knowledge of the biosynthetic pathway to vanillin

A knowledge of the genes involved in the biosynthetic pathway and its control

A selected host plant for the transformation

- Vanilla planifolia itself;
- Other plant species that make, but don't natural accumulate, vanillin (e.g. peppers);
- Species that don't naturally make vanillin but have commercial potential (e.g rice, corn);
- Model species to test out strategies (e.g Arabidopsis, tobacco);



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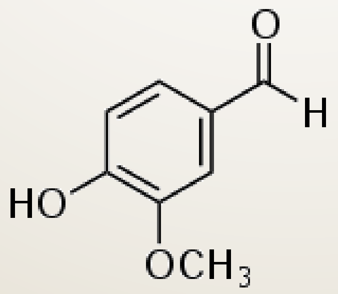
Compared to isoflavones and etoposides, vanillin is really simple. So why don't we understand how it is made?

- *Vanilla planifolia* is a genetically intractable crop
  - No efficient genetic transformation system for gene knock-down
  - No wide genetic variation
- Vanillin is made in specialized cell types and at specific times during development
- The fact that it is such a simple molecule means that it might be made by more than one route? This makes labeling studies difficult to interpret.
- It is hard to get funding to study vanillin biosynthesis (specialized product, non-US crop)
- To be honest, there are very few high-quality studies on vanillin biosynthesis (the best labs have avoided it?)



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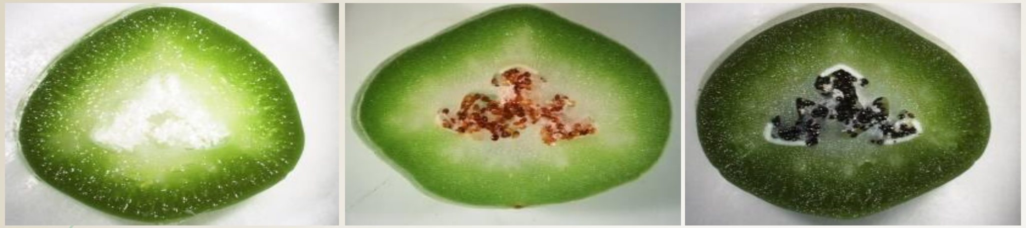
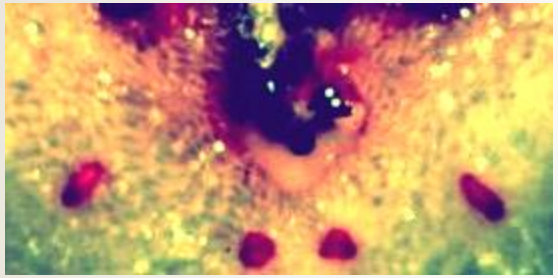
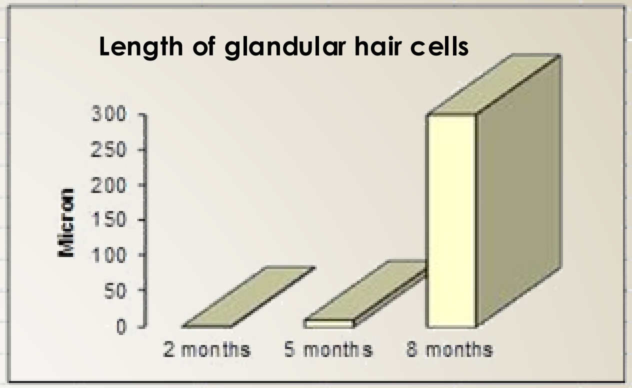
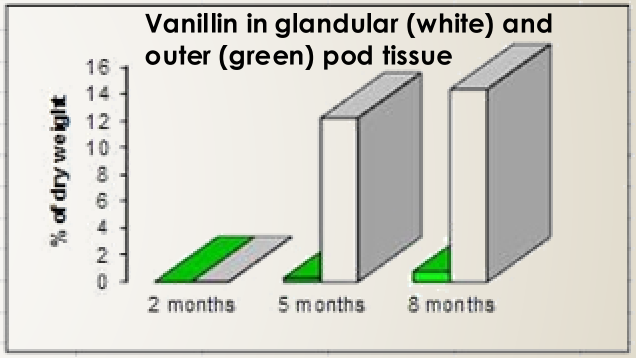
When and where is vanillin made?

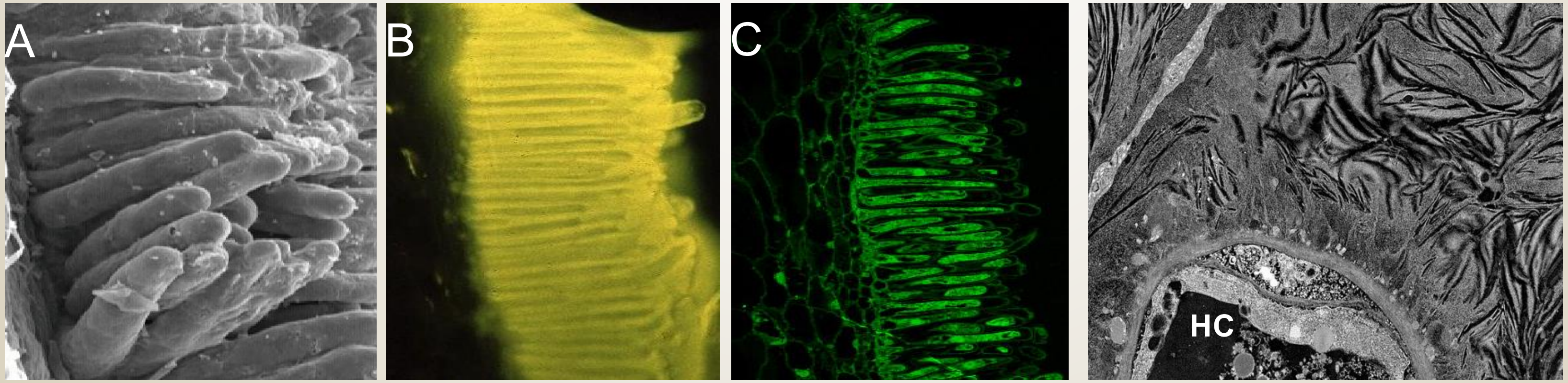


6 weeks 8 weeks 10 weeks









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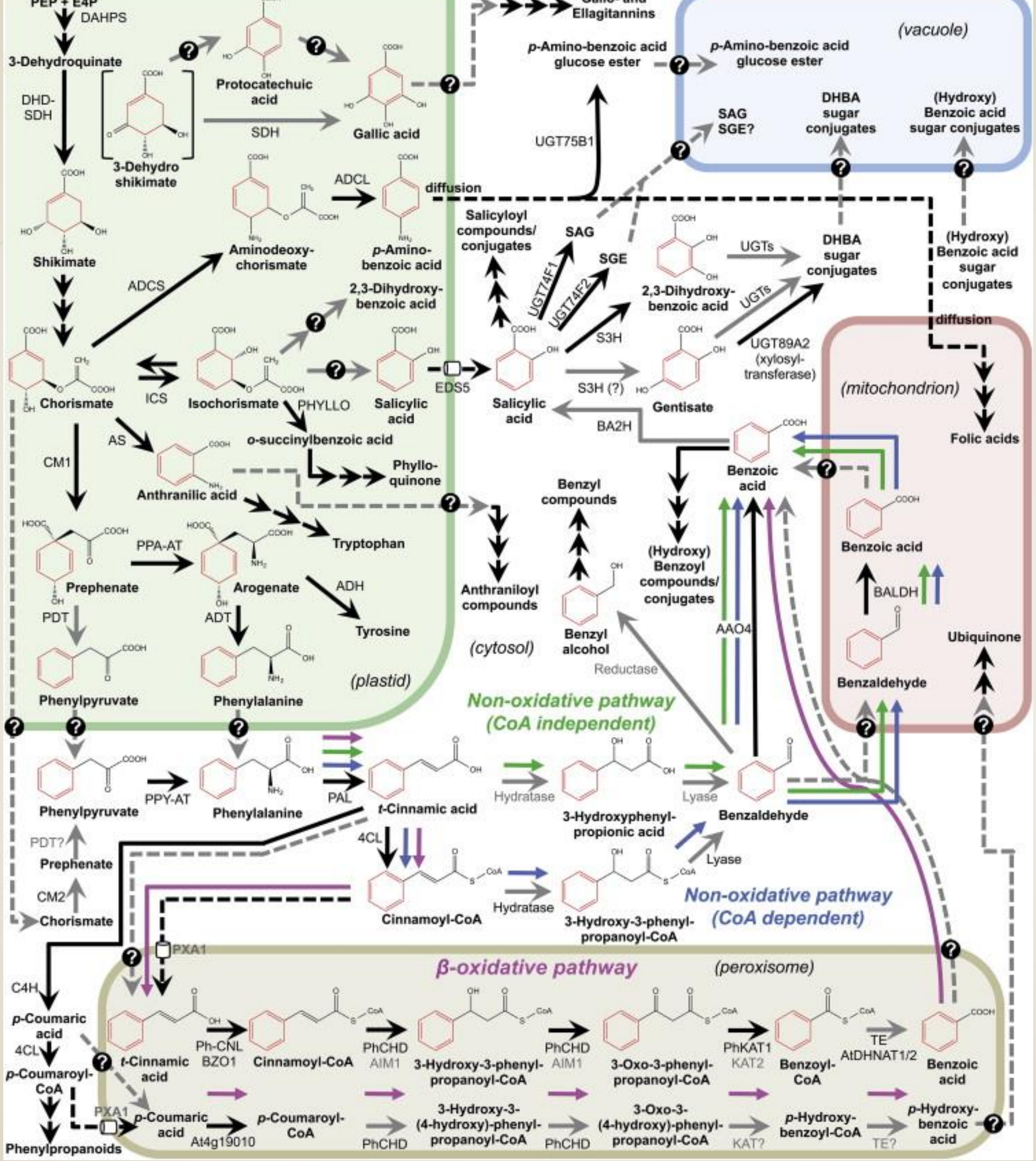

Widhalm and Dudareva, Molecular Plant, 2014

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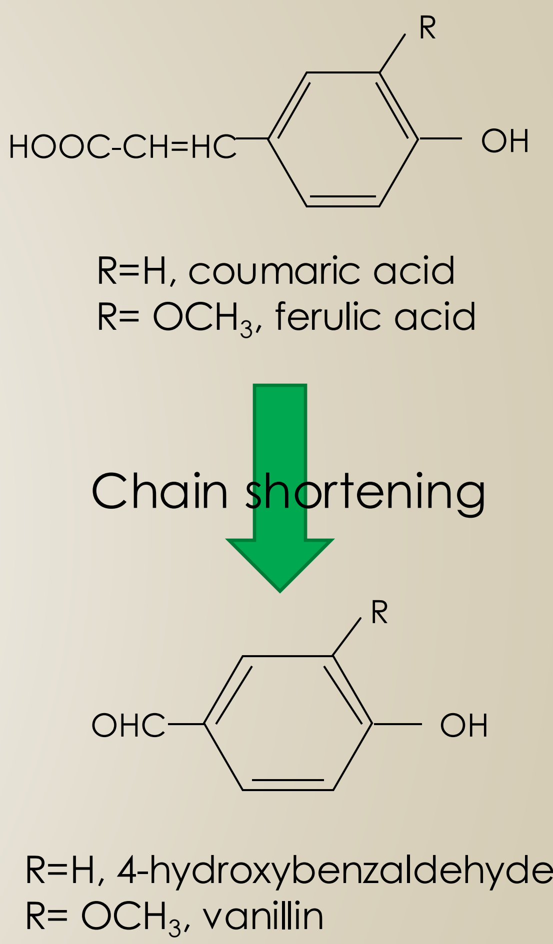
A brief history of research on vanillin biosynthesis

System and approach	Concept	Reference
Radiolabeling of V. planifola pods	Vanillin is formed directly from ferulic acid	Zenk, 1965
Radiolabeling of V. planifolia tissue cultures	Intermediacy of isoferulic acid (which is subsequently demethylated)	Funk and Brodelius, 1990a,b
Enzyme assay in cell free extracts from Lithospermum erythrorhizon	Non-oxidative chain-shortening of coumaric acid to 4-hydroxybenzaldehyde	Yazaki et al., 1991
Measuring metabolite levels in V. planifolia pods	Intermediacy of tartrate esters	Kanisawa et al., 1994
Enzyme isolation and assay from cell cultures of Hypericum androaemum	Involvement of a cinnamoyl CoA hydratase/lyase in non-oxidative chain shortening	El-Mawla et al., 2002
Enzyme isolation from V. planifolia cell cultures	Thiol-dependent non-oxidative conversion of 4-coumarate to benzaldehyde	Podstolski et al., 2002
Gene cloned from V. planifolia embryo cultures	A cysteine-protease like protein catalyzing weak thiol-dependent non-oxidative conversion of 4-coumarate to benzaldehyde	Havkin-Frenkel et al., 2013
Partial purification of an enzyme from pods of V. planifolia.	Iron-dependent dioxygenase for thiol-dependent chain-shortening of 4-coumaric and ferulic acids	Negishi and Negishi, 2014, 2015, 2017
Gene cloned from V. planifolia pods	Vanillin synthase, converting ferulic acid to vanillin; the same protein as in Havkin-Frenkel et al., 2013, above	Gallege et al., 2014



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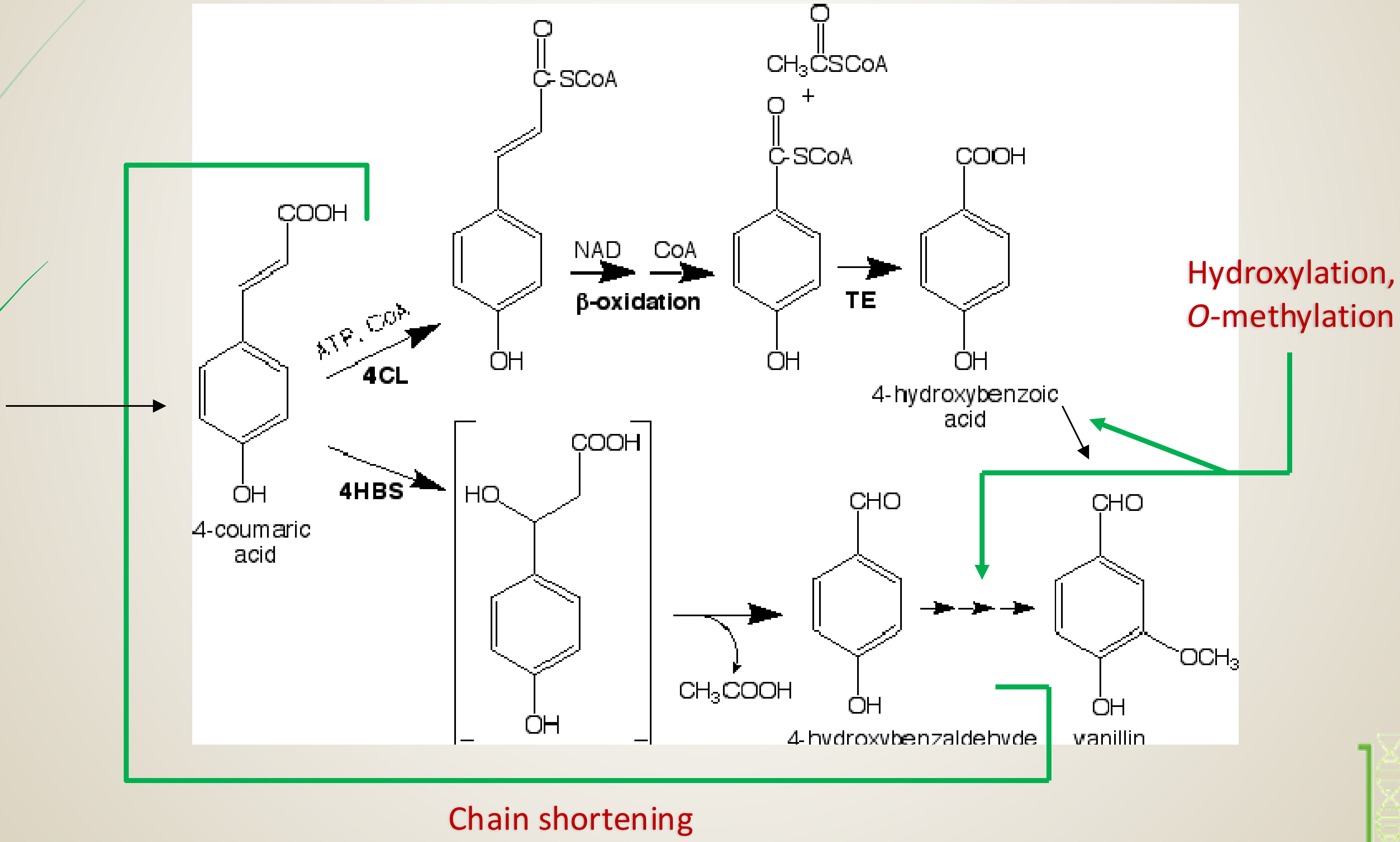
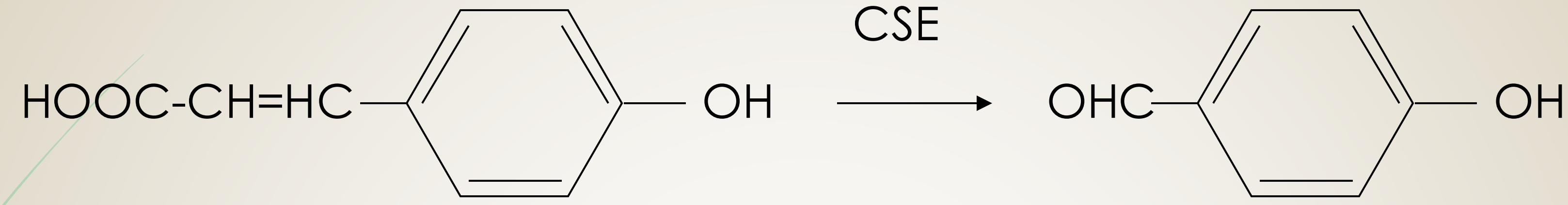
Two potential pathways for the chain-shortening reaction in the biosynthesis of vanillin in plants- oxidative and non-oxidative

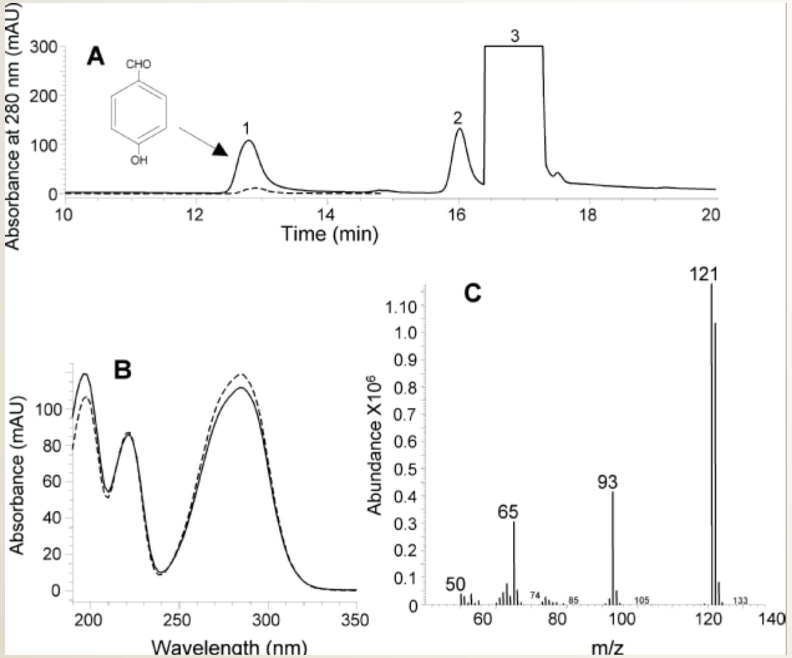
Example shows chain shortening of 4-coumaric acid, with subsequent hydroxylation and O-methylation. Alternatively, ferulic acid could be the substrate (chain shortening AFTER hydroxylation/O-methylation)

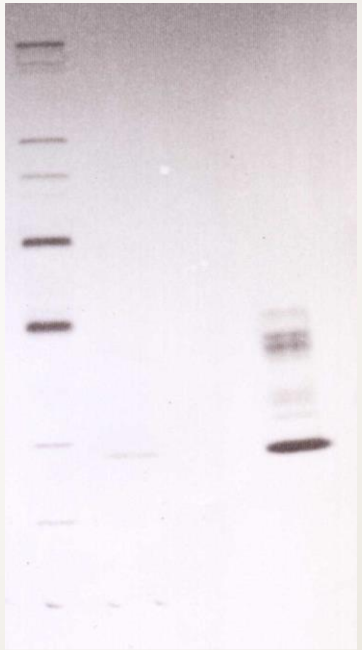


The old way- grind and find.

In vitro assay of the chain shortening enzyme from cell cultures of *Vanilla planifolia*







Sequence protein, clone gene

Cysteine protease-like protein (CPLP), with weak chain shortening activity with p-coumarate, virtually none with ferulate. No activity with ferulate in crude extracts

Podstolski et al., 2002
Havkin-Frenkel et al., 2003

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nature COMMUNICATIONS

ARTICLE

Received 19 Nov 2013 | Accepted 6 May 2014 | Published 19 Jun 2014 DOI: 10.1038/ncomms5037 OPEN

Vanillin formation from ferulic acid in *Vanilla planifolia* is catalysed by a single enzyme

Nethaji J. Gallage[1,2,3], Esben H. Hansen[4], Rubini Kannangara[1,2,3], Carl Erik Olsen[1,2], Mohammed Saddik Motawia[1,2,3], Kirsten Jørgensen[1,2,3], Inger Holme[5], Kim Hebelstrup[5], Michel Grisoni[6] & Birger Lindberg Møller[1,2,3,7]

Main conclusions of Gallege et al. 2014.

- Ferulic acid is the immediate precursor of vanillin
- A single enzyme, vanillin synthase (VS) converts ferulic acid to vanillin.
- VS is **identical** to an enzyme previously shown to be associated with formation of 4-hydroxybenzaldehyde
- VS is only found in the cells that make vanillin
- VS can be expressed in bacteria and other plants to allow them to make vanillin

The secret to "natural" vanillin through biotechnology?

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Phytochemistry 139 (2017) 33–46

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

ELSEVIER

A re-evaluation of the final step of vanillin biosynthesis in the orchid *Vanilla planifolia*

Hailian Yang [a,1], Jaime Barros-Rios [a,b], Galina Kourteva [c,2], Xiaolan Rao [a,b], Fang Chen [a,b], Hui Shen [a,3], Chenggang Liu [a,b], Andrzej Podstolski [d,4], Faith Belanger [e], Daphna Havkin-Frenkel [e], Richard A. Dixon [a,b,*]

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[b] Department of Biological Sciences, University of North Texas, Denton, TX 76203, USA
[c] Plant Biology Division, Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73402, USA
[d] Institute of Plant Experimental Biology, University of Warsaw, Miecznikowa 1, 02-096, Warsaw, Poland
[e] Department of Plant Biology, Rutgers, The State University of New Jersey, 59 Dudley Road, New Brunswick, NJ 08901, USA

We were unable to show formation of vanillin from ferulic acid when the enzyme was expressed in:

- In vitro transcription-translation system
- *E. coli*
- Yeast

Or formation of vanillin when the gene was expressed in :

- *Arabidopsis thaliana*
- *Medicago truncatula*

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Biochemical approaches didn't seem to be getting anywhere, so what about molecular biology?

RESEARCH ARTICLE Open Access

A deep transcriptomic analysis of pod development in the vanilla orchid (*Vanilla planifolia*)

Xiaolan Rao[1*], Nick Krom[2], Yuhong Tang[2], Thomas Widiez[3,4], Daphna Havkin-Frenkel[4], Faith C Belanger[4], Richard A Dixon[1] and Fang Chen[1*]

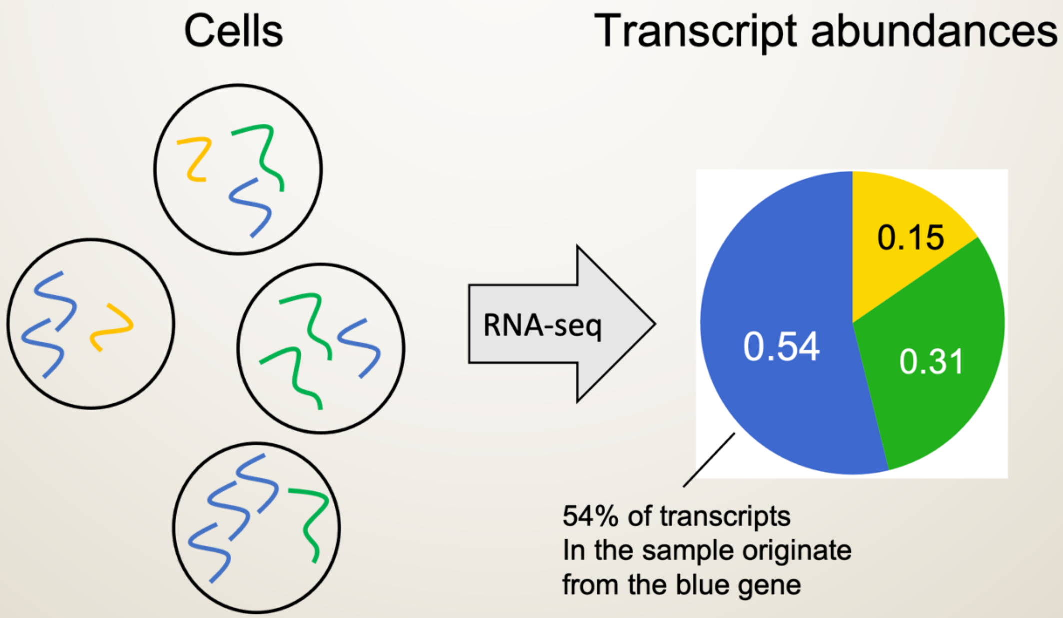
BMC Genomics (2014) 15: 964

Comparative transcriptome profiling of vanilla (*Vanilla planifolia*) capsule development provides insights of vanillin biosynthesis

Manuel Gastelbondo[1], Vincent Micheal[2], Yu Wang[3], Alan Chambers[4] and Xingbo Wu[1,2*]

BMC Plant Biology (2025) 25:343

Compares two accessions with different vanillin levels. Identifies PAL, COMT, CPLP as having higher expression in line with higher vanillin.



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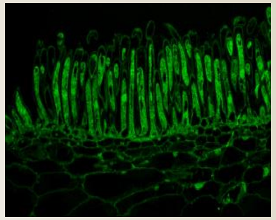
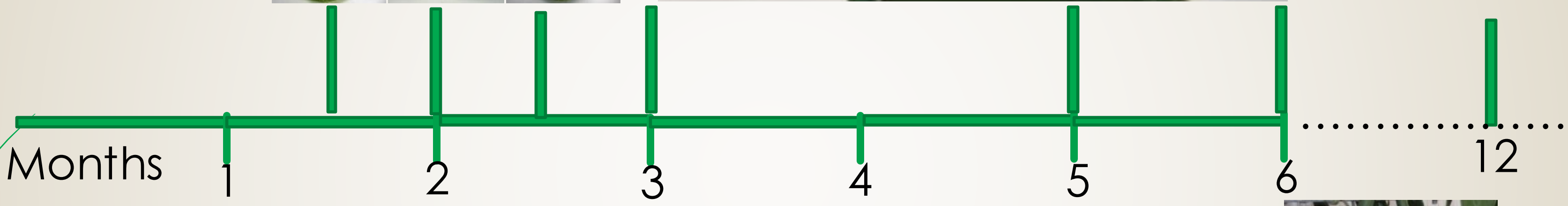
Harvesting Vanilla tissues for RNA sequencing

Seed, V-shaped *papillae*, placental *laminae*, and mesocarp tissues



Seed, placental *laminae* and mesocarp



RNA was extracted and cDNA prepared from 26 different tissue samples consisting of different tissues from early stages of pod development (seed, placental *laminae* and mesocarp tissues at 6 , 8 and 10 weeks post-pollination), mature pods (seed, V-shaped *papillae*, placental *laminae*, and mesocarp tissues at 3, 5 and 6 months post-pollination), leaf and stem at 6 months and 1 year post-pollination and aerial roots harvested at 1 year.

Leaf, stem, aerial root

Rao et al. 2014

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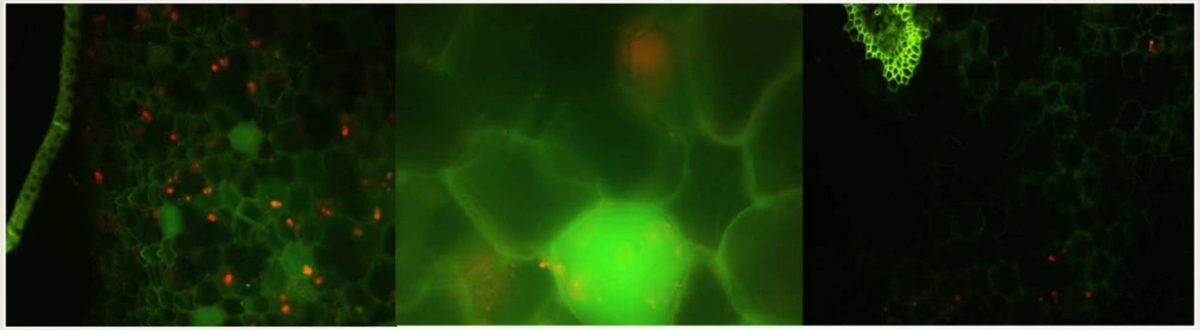
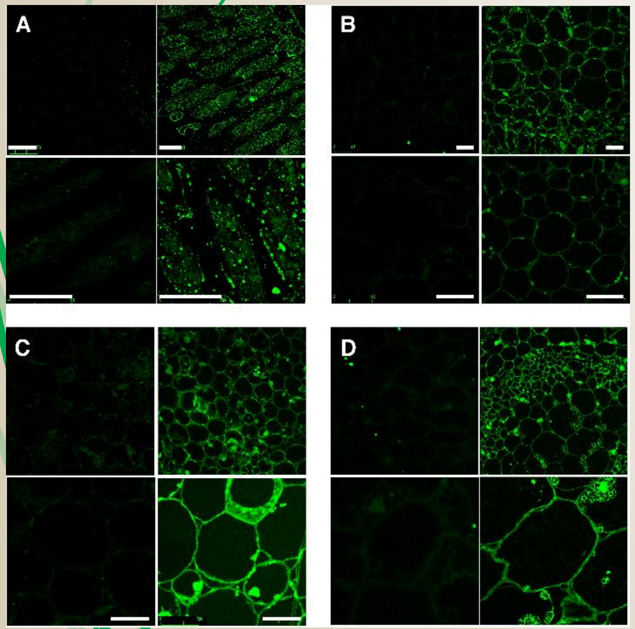
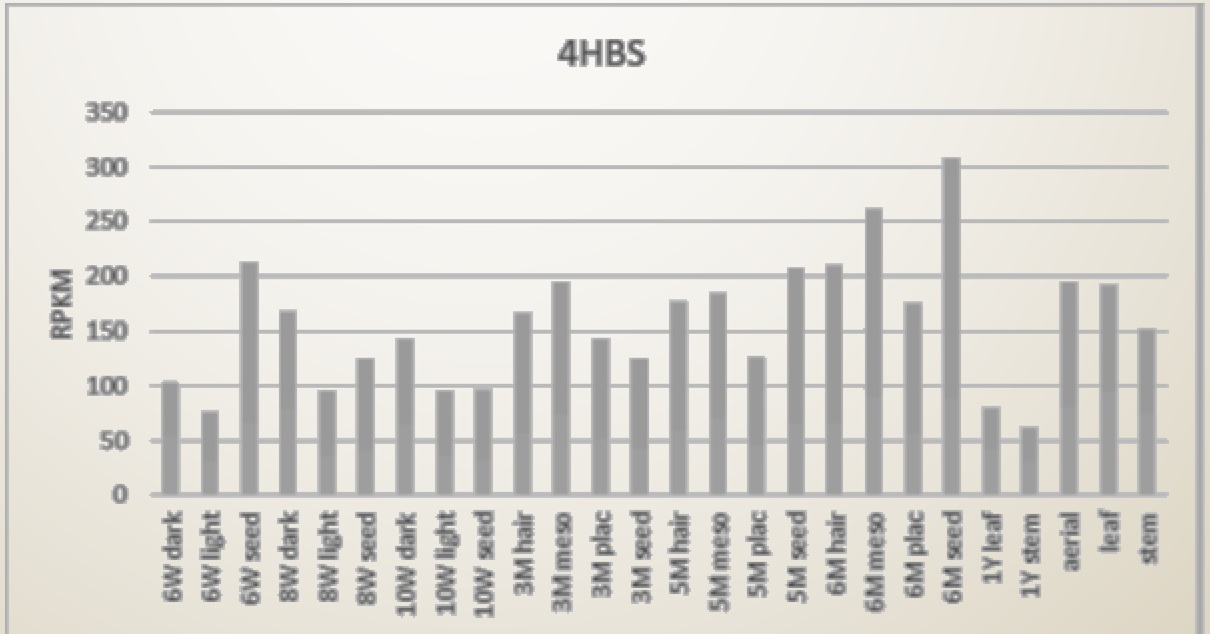
Controversy as to the location of the CPLP

Gallege et al., 2024. "Vanillin synthase" is only found in the cells that make vanillin-inner part of pod only



Yang et al., 2017. The cysteine-protease-like protein ("VS") is made and found in all cell types and tissues





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What next?

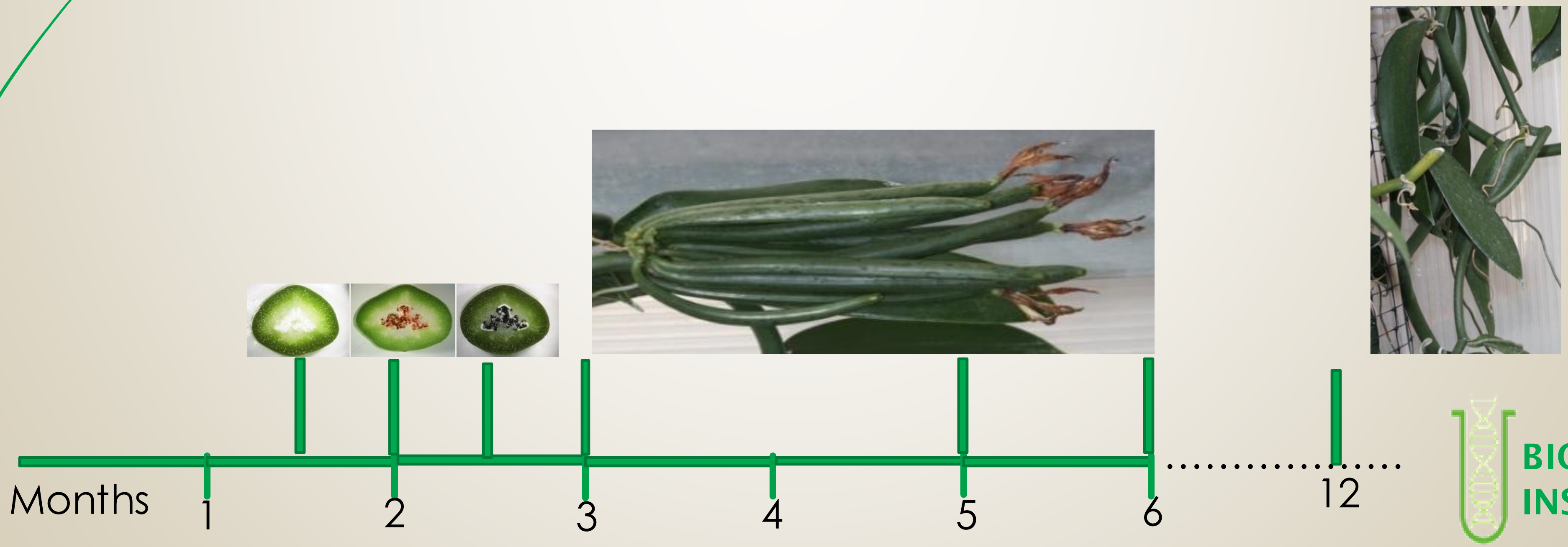
Re-evaluate the early labeling experiments

- How? Use ^{13}C-labeled precursors in cell cultures and beans

Evaluate other candidate genes from the RNAseq database

- Express in bacteria/yeast?
    - Really need a quick transient assay system for *V. planifolia*, but could perhaps use tobacco for gain-of-function experiments
    - Need a robust loss of function system in *V. planifolia*. This is the big problem.

Work on other plant systems that make vanillin?



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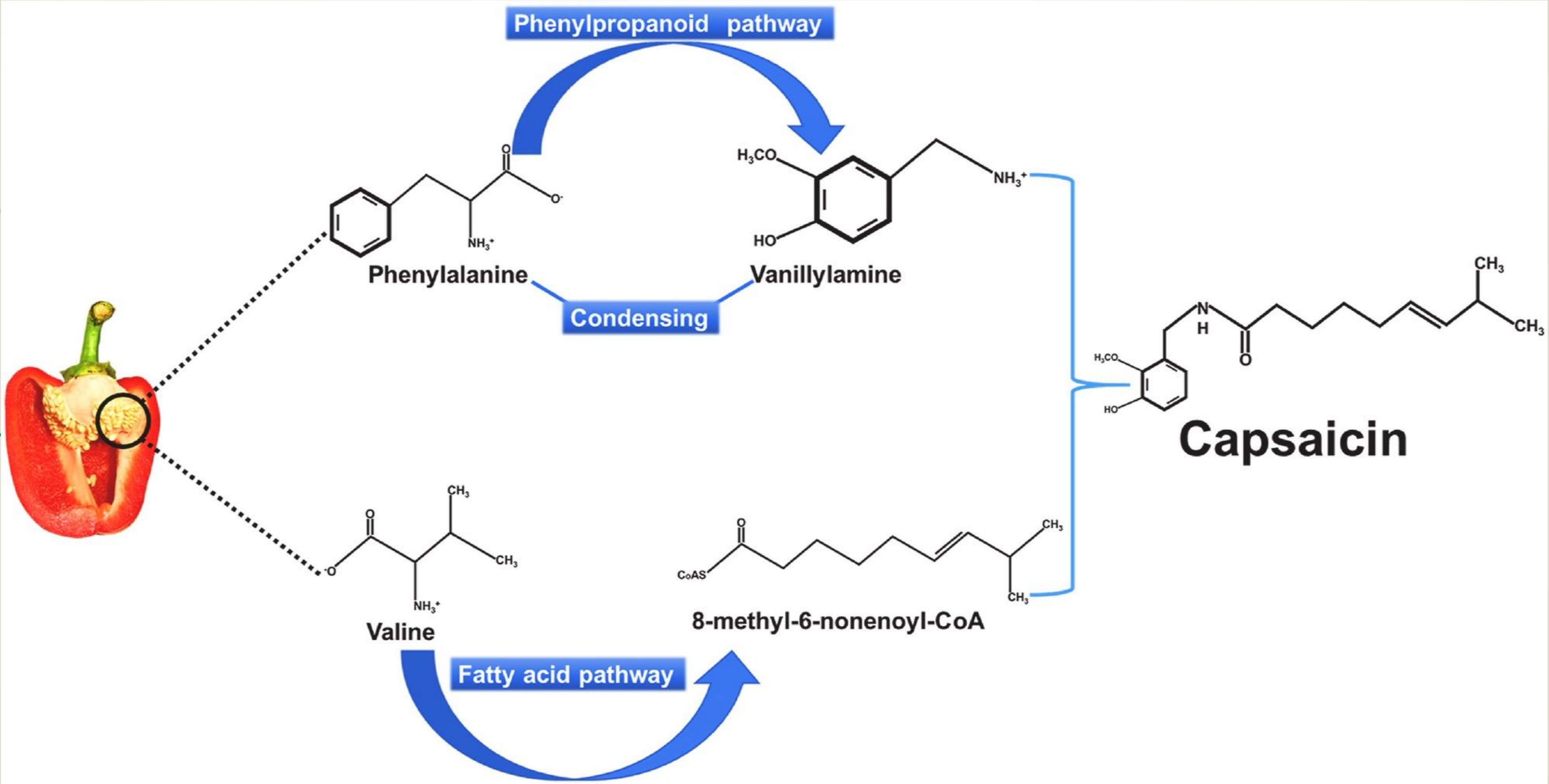
Engineering vanillin in pepper?



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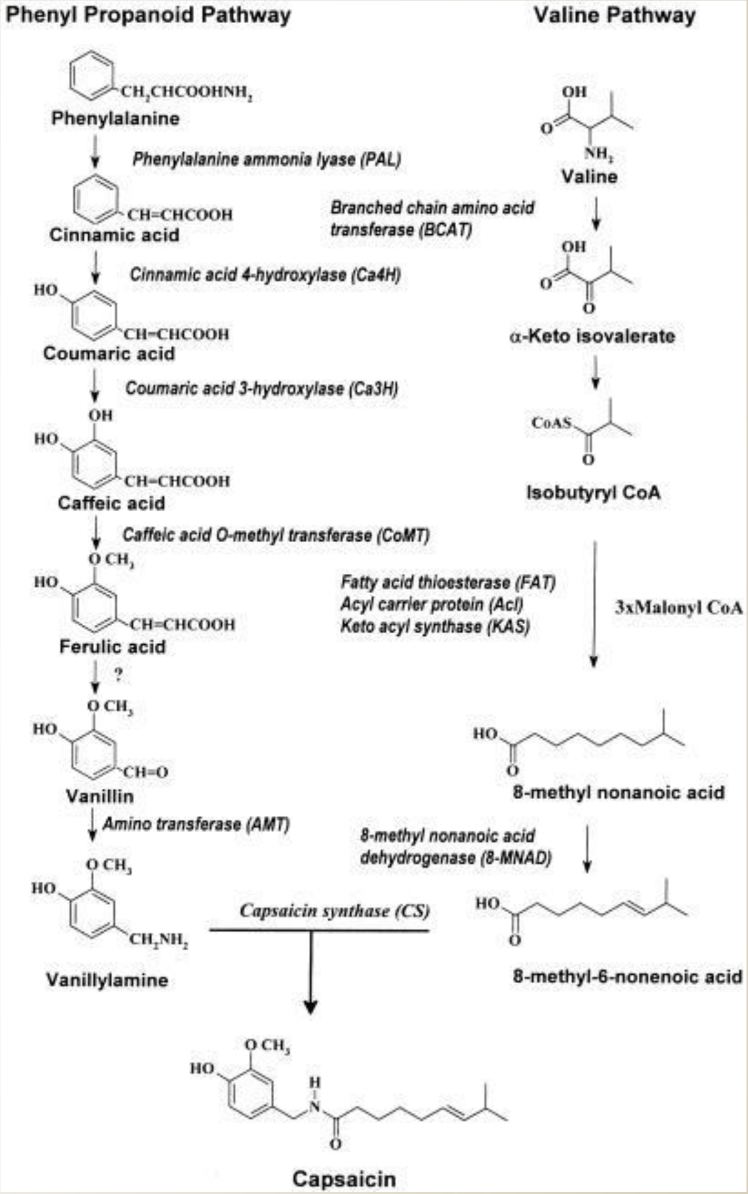
The pathway to vanillin in pepper is still not clear

B.C. Narasimha Prasad, V. Kumar, H.B. Gururaj, R. Parimalan, P. Giri dhar, G.A. Ravishankar
Characterization of capsaicin synthase and identification of its gene (cys1) for pungency factor capsaicin in pepper (*Capsicum* sp.)
Proc. Natl. Acad. Sci. U. S. A., 103 (2006), pp. 13315-13320

"Subsequent to our publication it was pointed out to us by a reader that the *csy1* sequence showed homology with the leucine rich putative protein kinase (EF560217) that appeared post publication of our paper. Accordingly, the identified gene cloned in our article does not conclusively encode to capsaicin synthase and we must retract the PNAS paper."

Ogawa, K., Murota, K., Shimura, H. *et al.* Evidence of capsaicin synthase activity of the *Pun1*-encoded protein and its role as a determinant of capsaicinoid accumulation in pepper. *BMC Plant Biol* **15**, 93 (2015). https://doi.org/10.1186/s12870-015-0476-7

Zhang, ZX., Zhao, SN., Liu, GF. *et al.* Discovery of putative capsaicin biosynthetic genes by RNA-Seq and digital gene expression analysis of pepper. *Sci Rep* **6**, 34121 (2016). https://doi.org/10.1038/srep34121



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Engineering vanillin in heterologous species using "VpVAN/CPL"

Chee, M.J.Y., Lycett, G.W., Khoo, TJ. *et al.* (2017) Bioengineering of the plant culture of *Capsicum frutescens* with vanillin synthase gene for the production of vanillin. *Molecular Biotechnology* **59**, 1–8. Problem with identification of vanillin; no changes in ferulic acid; just 3 lines, no statistics, but BIG increase in "vanillin".

Arya, S.S., Mahto, B.KJ., Sengar, M.S. (2022). Metabolic engineering of rice cells with *Vanillin Synthase* gene (*VpVAN*) to produce vanillin. *Molecular Biotechnology*. https://doi.org/10.1007/s12033-022-00470-8. Problem with identification of vanillin in cell clump cultures. No MS data.

Husain, Z., Khan, S. , Sarfraz, A. et al. (2024) Metabolic-engineering approach to enhance vanillin and phenolic compounds in *Ocimum sanctum* (CIM-Angana) via VpVAN overexpression. *Physiologia Plantarum* 176:e70005. All pathway enzymes were induced; no changes in ferulic acid, but 4-hydroxybenzyl alcohol levels increase in parallel with vanillin. Large variation in samples.

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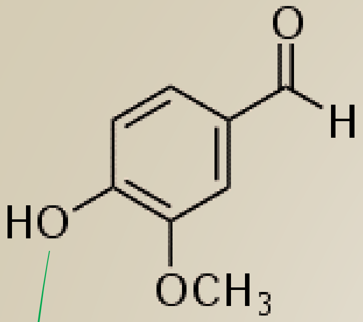
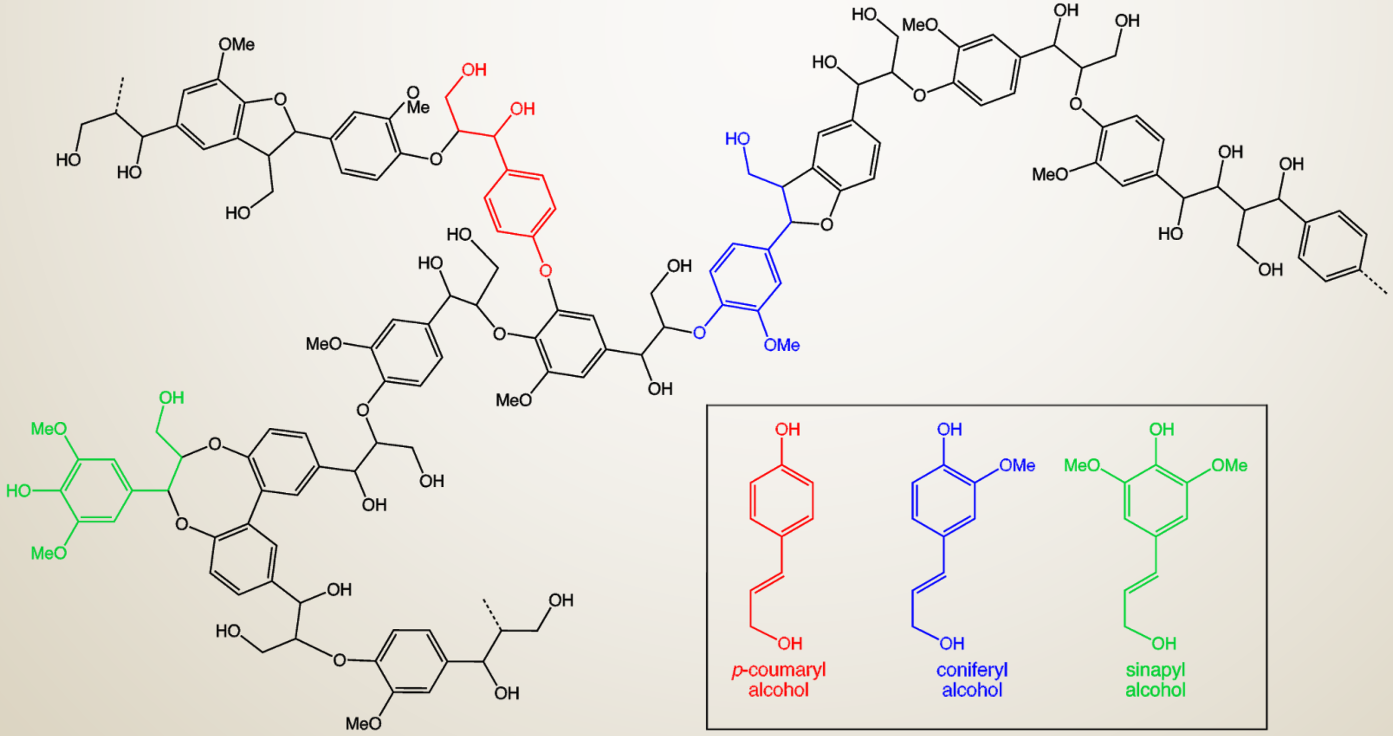


Making vanillin from lignin

A complex polymer, the chief non-carbohydrate constituent of wood, that binds to cellulose fibers and hardens and strengthens the cell walls of plants.---
American Heritage Dictionary



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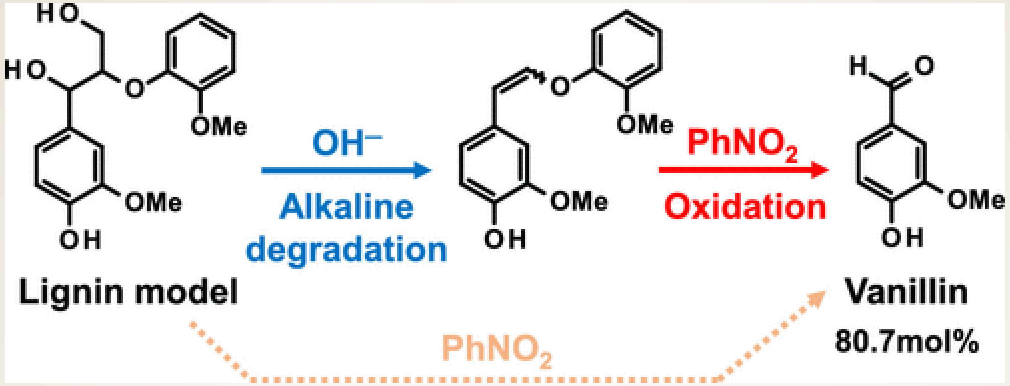
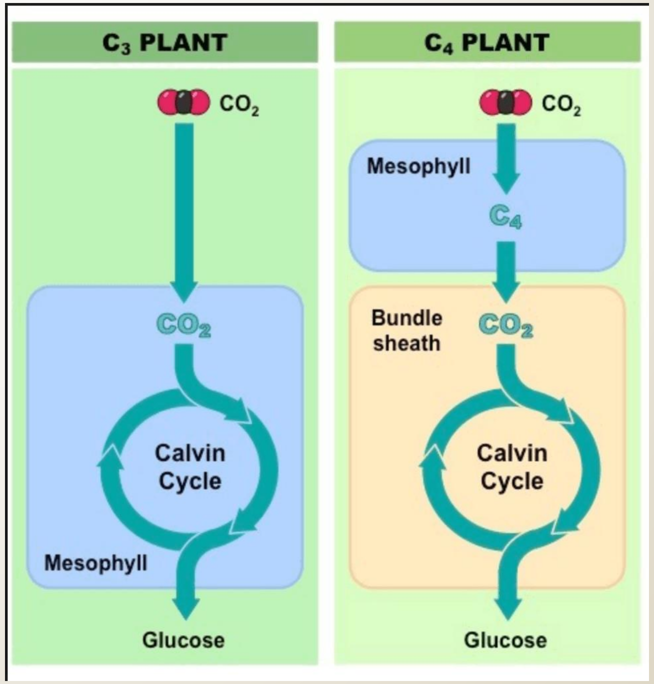
Alkaline nitrobenzene oxidation of lignin has been known for many years, and is used as an analytical tool



$^{12}C/^{13}C$ ratio will tell whether vanillin has come from a monocot (e.g. V. planifolia) or dicot plant (e.g. poplar).



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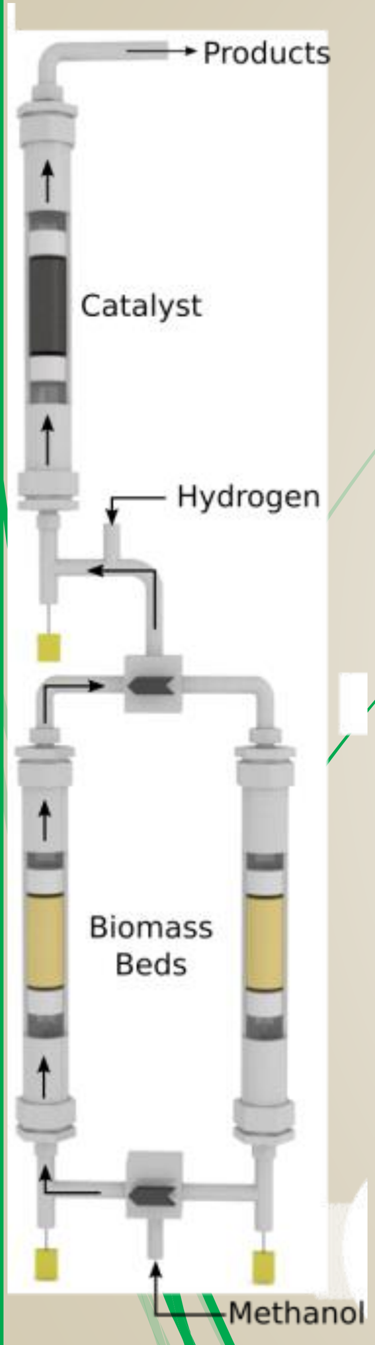
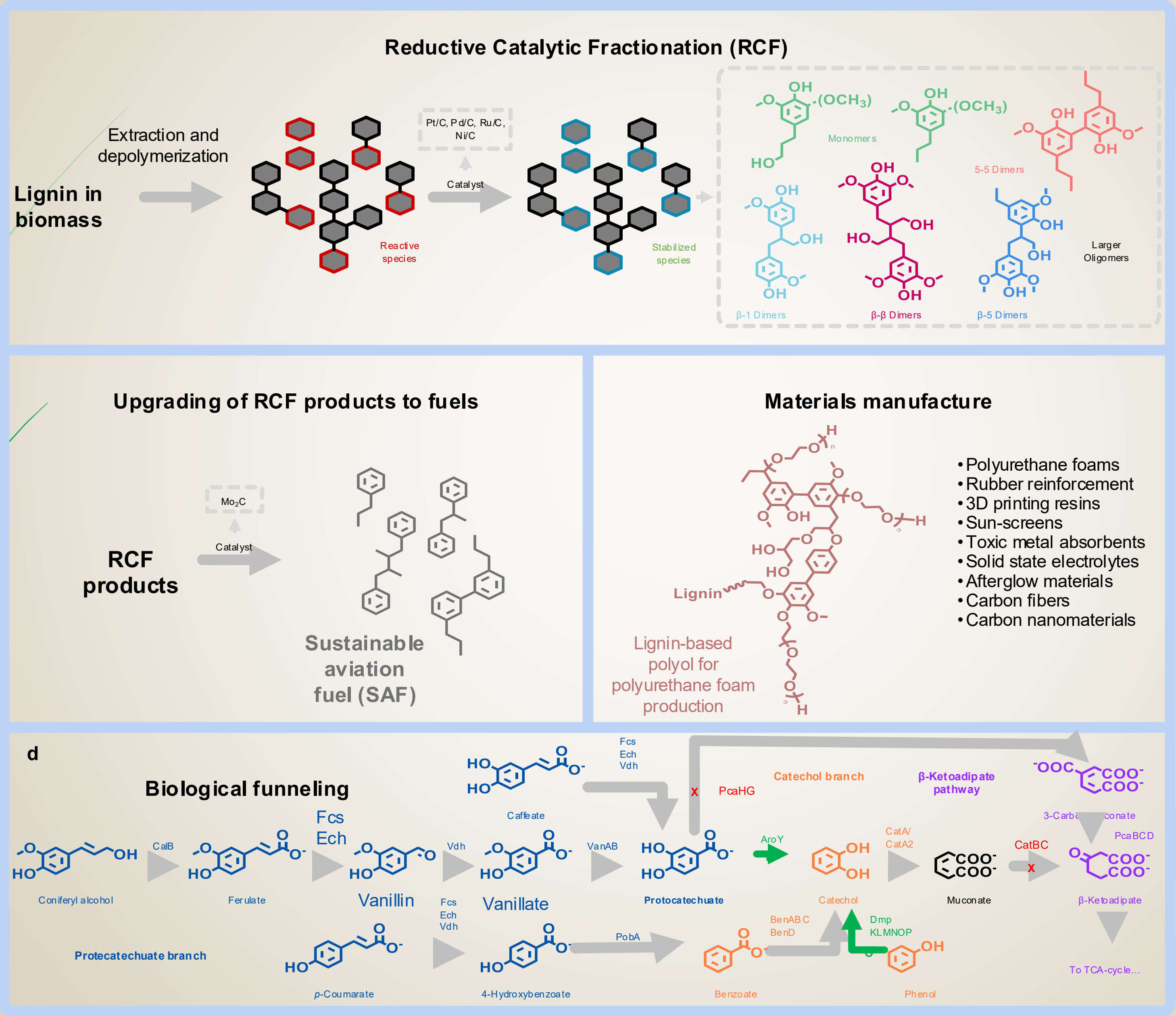
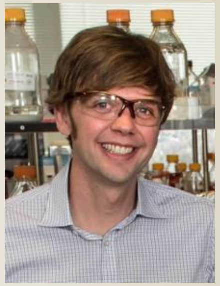
Lignin valorization

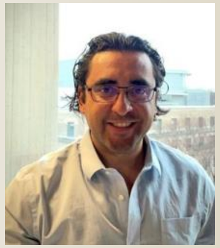




Dixon et al.. Annual Review of Plant Biology 75: 239-263.

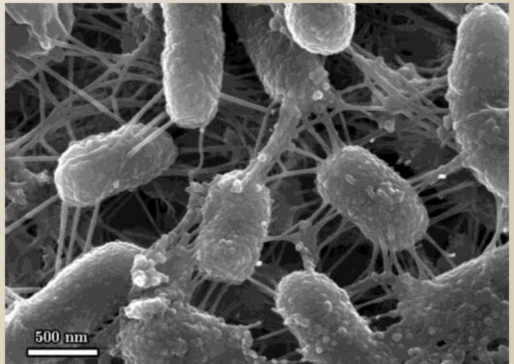


Gregg Beckham (NREL)



Yuriy Roman (MIT)

Pseudomonas putida



Thanks to:

Samuel Roberts Noble Foundation; National Science Foundation
USDA; US Department of Energy; NIH

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