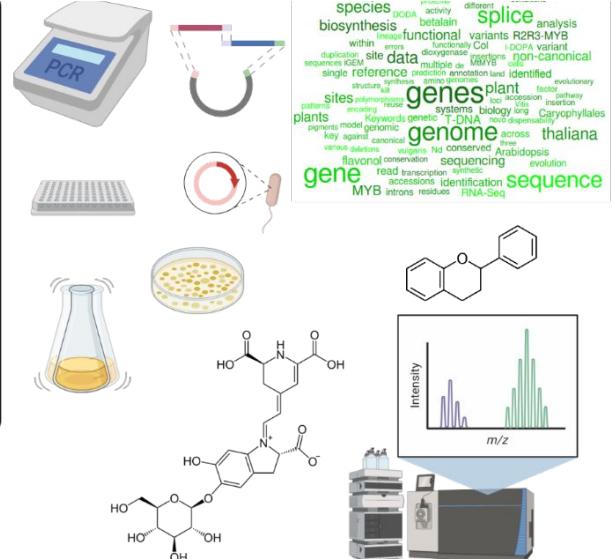
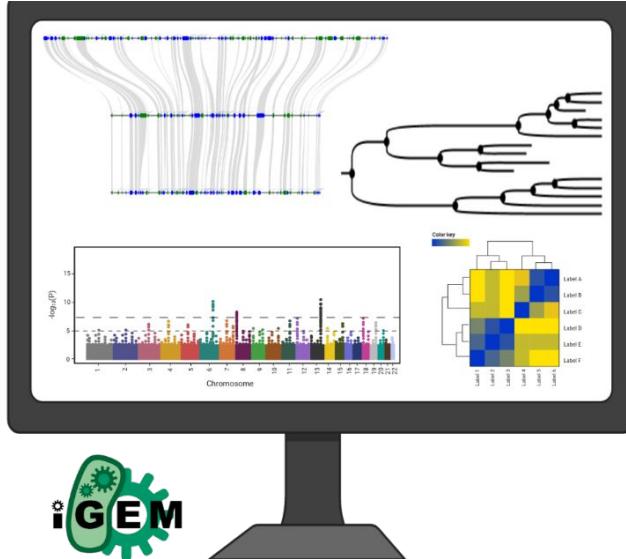
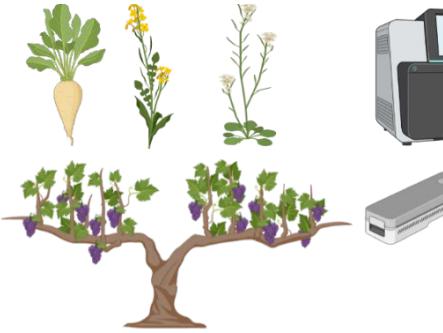




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Identification of biosynthesis pathways

Prof. Dr. Boas Pucker (Plant Biotechnology and Bioinformatics)

Availability of slides

- All materials are freely available (CC BY) - after the lectures:
 - StudIP: [Lecture: Grundlagen der Biochemie und Bioinformatik der Pflanzen \(Bio-MB 09\)](#)
 - Skype: (link shared via email)
 - GitHub: <https://github.com/bpucker/teaching>
- Questions: Feel free to ask at any time
- Feedback, comments, or questions: b.pucker@tu-braunschweig.de



My figures and content can be re-used in accordance with CC BY 4.0, but this might not apply to all images/logos. Some figure were constructed using bioRender.com.

Do you know metabolites produced by plants?



Specialized plant metabolites



Gossypium davidsonii
cellulose (cotton cloth)



Daucus carota
anthocyanins (food color)

Erythroxylum coca
cocaine (local anesthesia)

Cinchona officinalis
quinine (malaria)



Artemisia annua
artemisinin (malaria)



Beta vulgaris
betalains (food color)



Zea mays
 β -carotene (provitamin A)

Taxus brevifolia
paclitaxel (cancer)

Applications of plant metabolites

Atropa belladonna
scopolamine (motion sickness)

Chrysanthemum cinerariaefolium
pyrethrin I (insect control)

Digitalis purpurea
digoxin (congestive heart failure)



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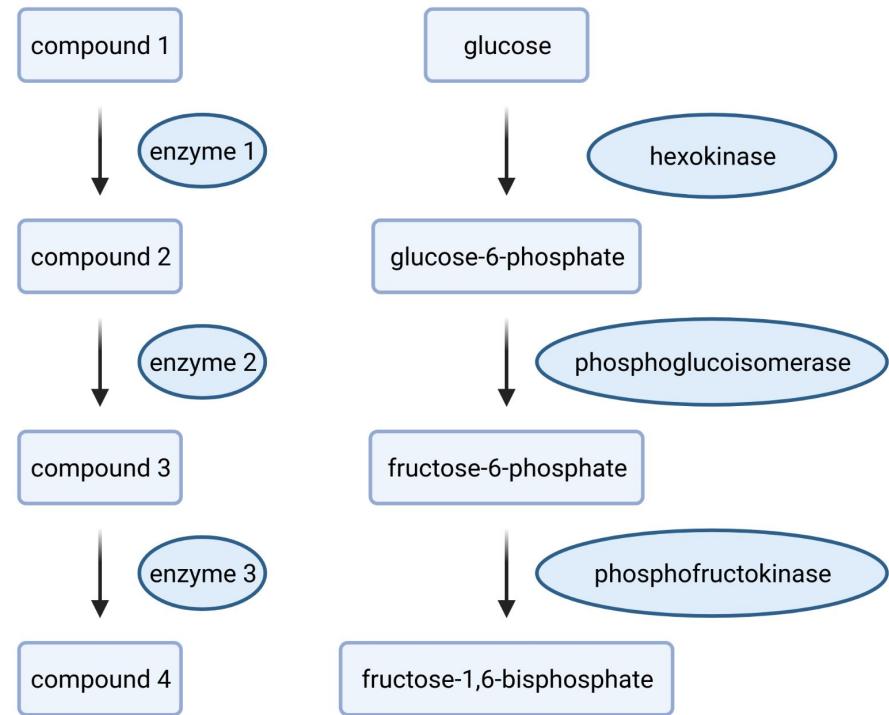
Rauwolfia serpentina
ajmaline (cardiac arrhythmia)

Crocosmia × crocosmiiflora
montbretin A (diabetes)

Partly inspired by 10.1126/science.aad2062
Irmisch et al., 2020: 10.1104/pp.20.00522

Biosynthesis pathways

- Biosynthesis pathways comprise steps catalyzed by different enzymes
- Chemical compounds are substrates/products of multiple steps
- How can we understand individual enzyme functions?

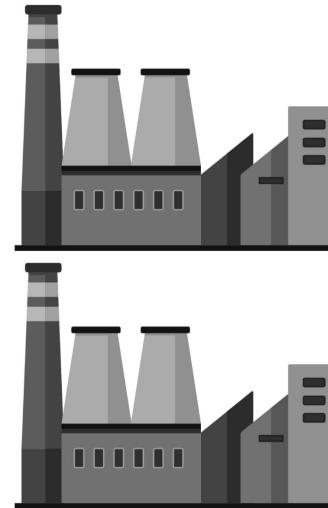


How would you identify the function of a gene?



How to understand the function of a gene/enzyme?

- How would you find out what a car factory worker is doing?
- Bind/remove hands and arms and see how the product differs from the normal product
- Transfer to gene: knock-out a gene and see how the plant looks different compared to a wild type



wild type

mutant

How can we do this in plants?

- How to knock-out a plant gene?
- Targeted knock-out through homologous recombination is impossible
- Random mutagenesis was method of choice for decades of plant research:
 - T-DNA insertions
 - transposons
 - chemical mutagenesis (EMS)
 - radiation
- CRISPR-Cas

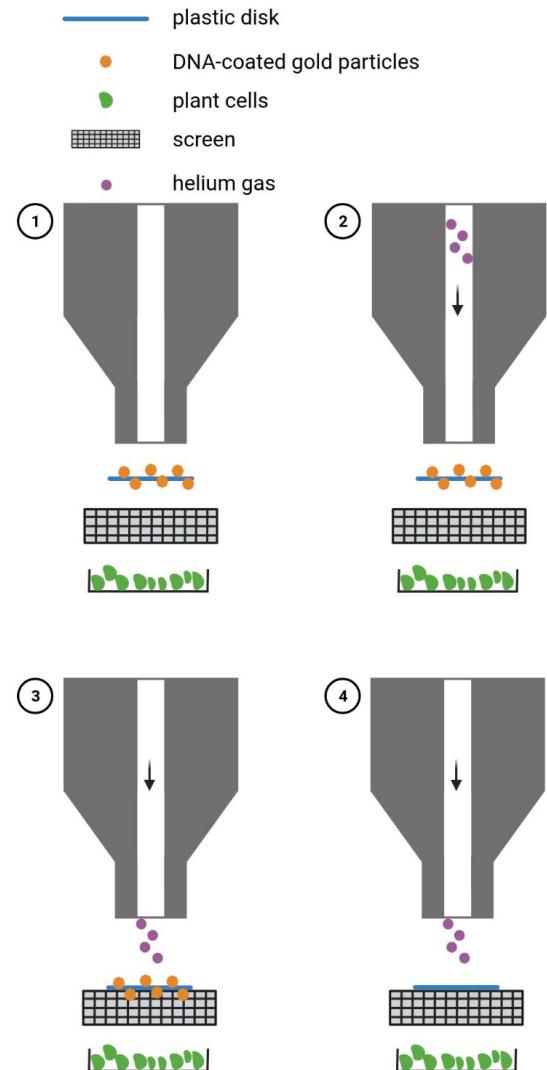


Integrating genes in plants

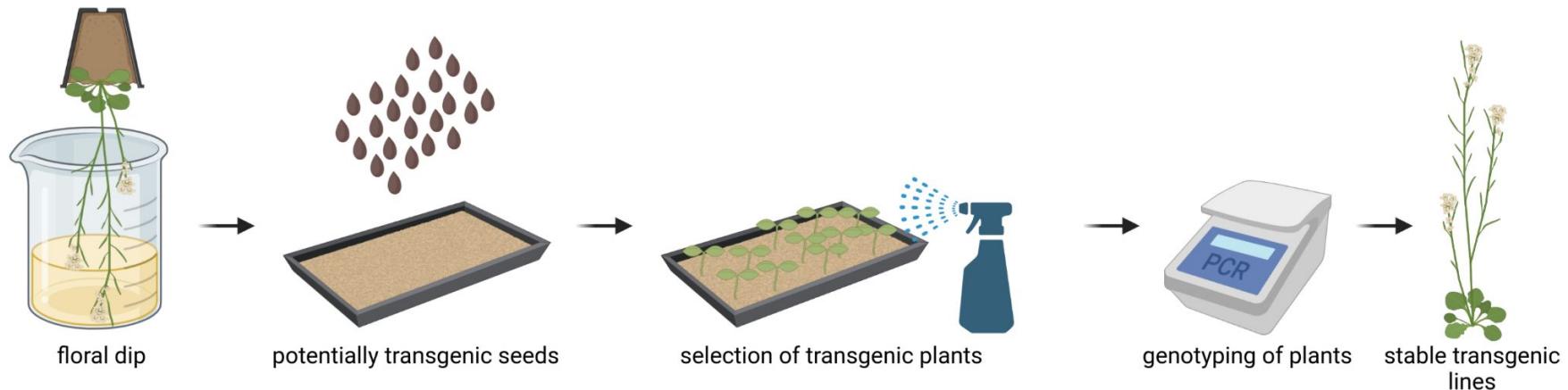
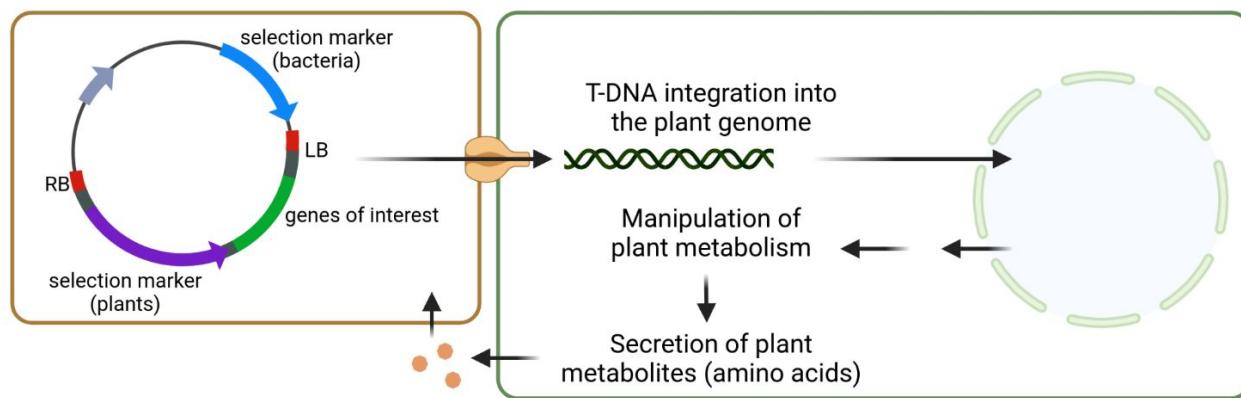
- Methods for stable transformation:
 - particle gun
 - Agrobacterium-mediated
- Integration of DNA in the genome is almost random (no effective homologous recombination)
- Transient transfection of protoplast can be done through CaCl_2 -based methods or electroporation

Particle gun

- Shooting DNA into plant cells
- Applicable to all plant species
- Optimization of conditions required

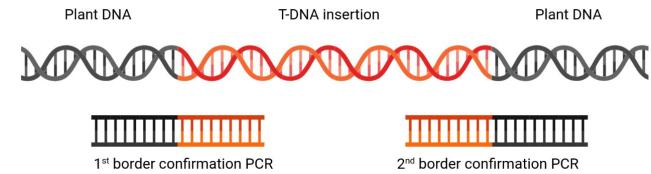
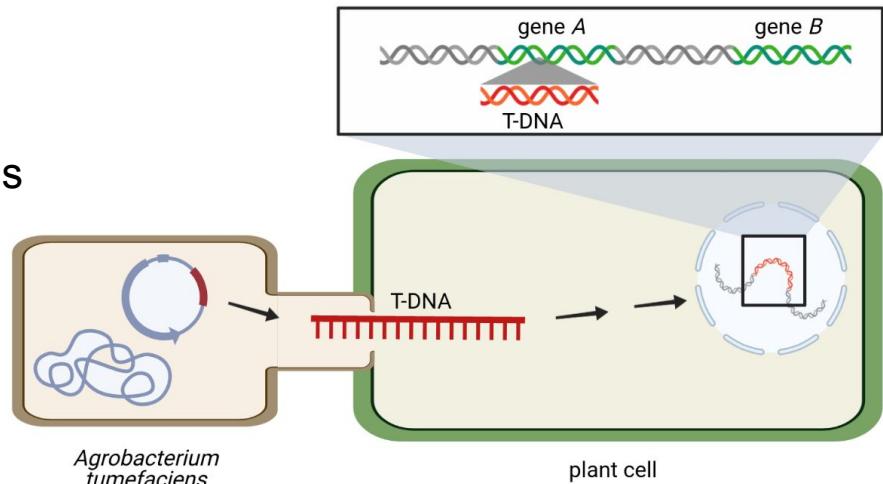


Agrobacterium-mediated gene transfer (floral dip)



T-DNA

- Integration of DNA in the genome is almost random (no effective homologous recombination)
- T-DNA can disrupt genes
- T-DNA carries marker for selection of transgenic plants



Transposon (Ac/Ds)

- Activator/Dissociation(Ac/Ds) originates from maize
- Ac is transposase that moves the Ds element

Ds-GUS T-DNA:



uidA = beta-glucuronidase (blue coloration)

aph4 = aminocyclitol phosphotransferase

■ Ac-derived sequences

35S-Ac T-DNA:



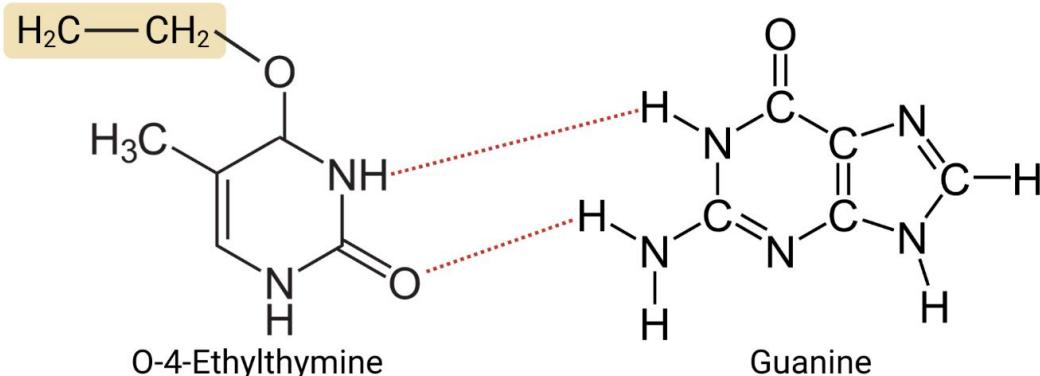
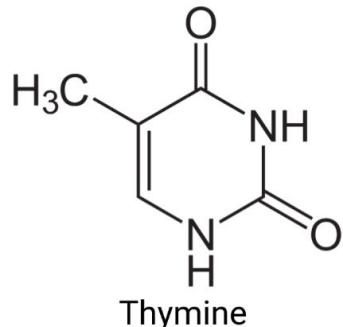
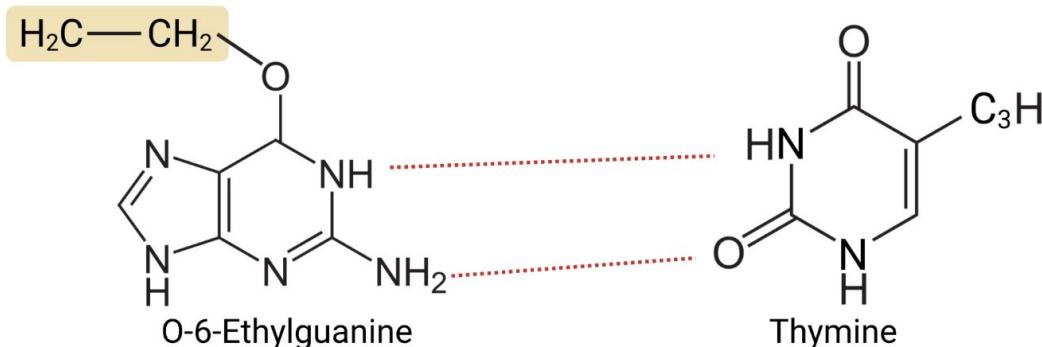
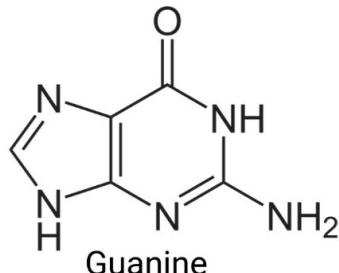
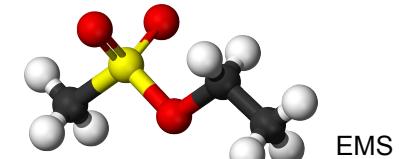
tms2 = indolacetamide hydrolase (conditionally lethal)



https://en.wikipedia.org/wiki/File:Corn_3different_types.jpg

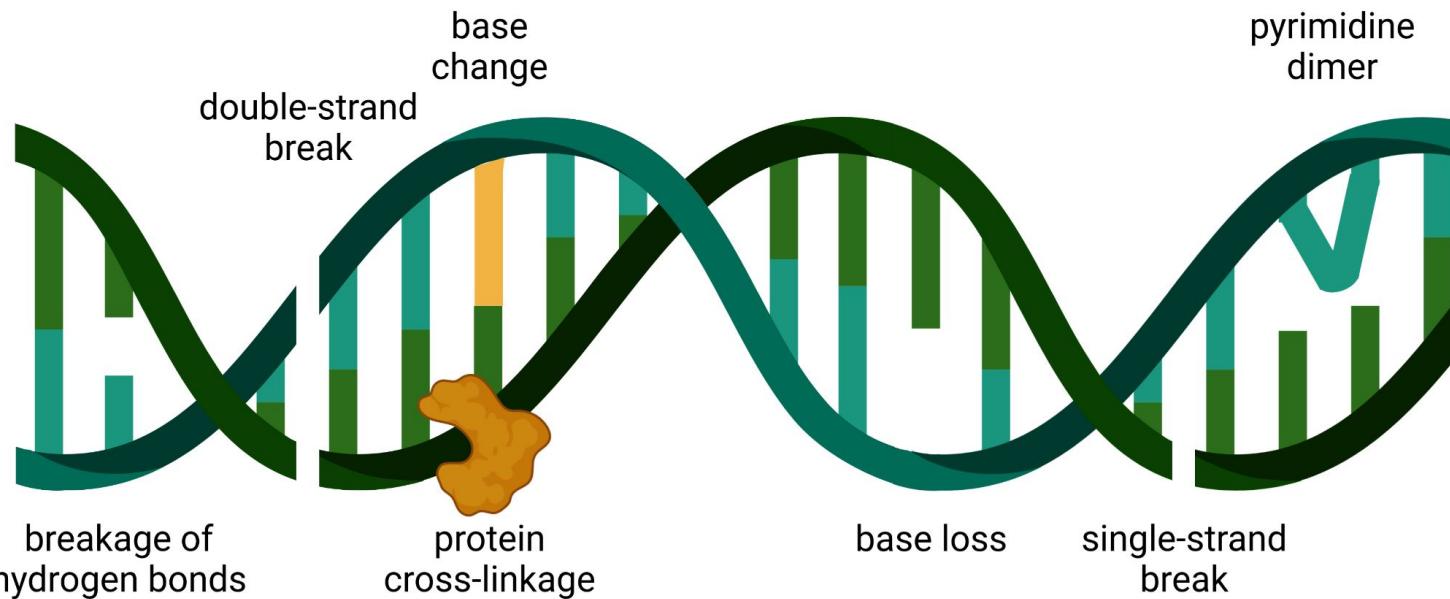
Ethyl methanesulfonate (EMS)

- EMS causes mostly point mutations
- Seeds are incubated in EMS for mutagenesis



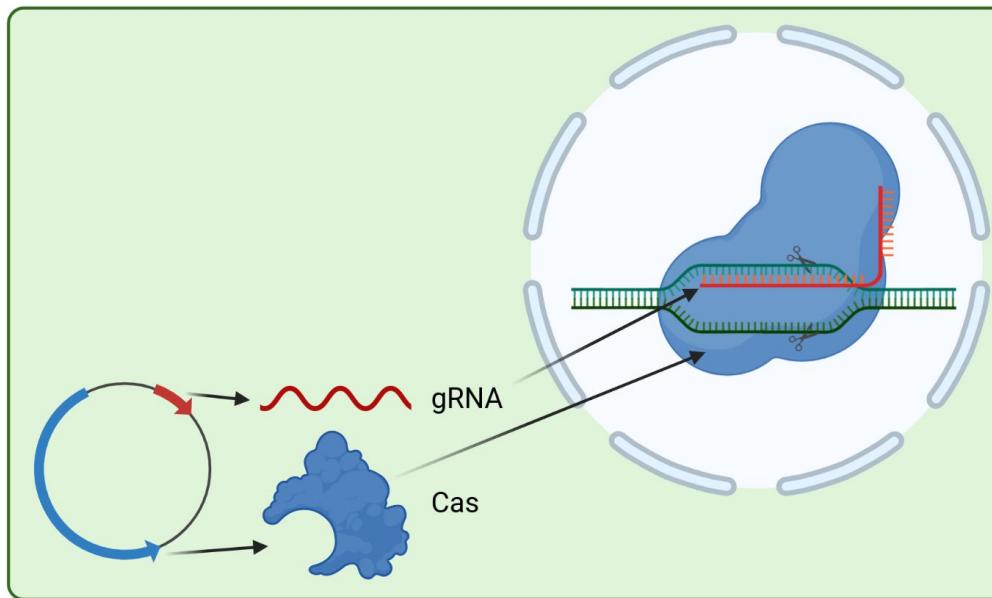
Radiation

- Energy-rich radiation has a diverse range of effects on DNA
- Example: gamma, UV



Opportunities using CRISPR/Cas9

- CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats
- Targeted modification in plant genomes
- Modifications:
 - dCAS (inactive) can be used to block promoter
 - Methyltransferases can be attached to Cas protein
 - ...



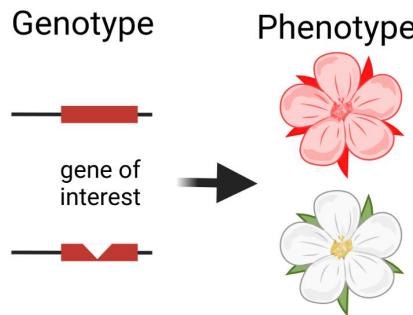
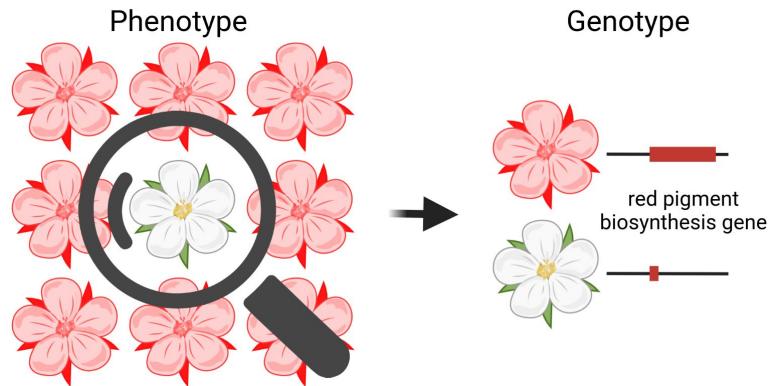
'Emmanuelle Charpentier and Jennifer A. Doudna have discovered one of gene technology's sharpest tools: the CRISPR/Cas9 genetic scissors. Using these, researchers can change the DNA of animals, plants and microorganisms with extremely high precision. This technology has had a revolutionary impact on the life sciences, is contributing to new cancer therapies and may make the dream of curing inherited diseases come true.'

<https://www.nobelprize.org/prizes/chemistry/2020/press-release/>



Forward and reverse genetics

- Forward genetics = Interesting phenotype is observed and responsible gene is identified in the next step
 - Examples: transposon mutagenesis, EMS, radiation
- Reverse genetics = Finding the function of a known gene through targeted mutation of this gene
 - Examples: T-DNA collections, CRISPR-Cas



Which properties of a model species do you know?



Model systems - species

- Investigation of a research question requires a model species
- Important properties of model species:
 - Fully sequenced genome
 - Small size
 - Large number of seeds (offspring)
 - Accessible by genetic engineering
 - Short generation time
 - Large community
 - ...

*Arabidopsis
thaliana*



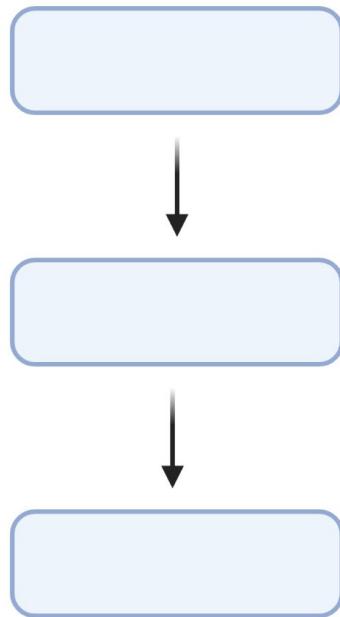
https://commons.wikimedia.org/wiki/File:Arabidopsis_thaliana.jpg (CC-BY-SA)

Model system - species (examples)

- *Arabidopsis thaliana* (thale cress): THE most important model system due to the genome sequence
- *Antirrhinum majus* (snapdragon): pioneer model plant; variation and genetics
- *Petunia x hybrida* (petunia): evolution and development
- *Zea mays* (maize): discovery of transposons by Barbara McClintock (Nobel prize)
- *Oryza sativa* (rice): model organism for research on crops

Parry et al., 2020: 10.1002/pld3.248
Schwarz-Sommer et al., 2003: 10.1038/nrg1127
Vandenbussche et al., 2016: 10.3389/fpls.2016.00072
Strable & Scanlon, 2009: 10.1101/pdb.em0132
Rensink & Buell, 2004: 10.1104/pp.104.040170

What are the important properties of a model biosynthesis pathway?



Model system - pathway

- Easily detectable product
- Stable intermediates that can be quantified
- Gene knock-outs must not be lethal
- Conserved across many plant species
- Community working on the pathway
- Different branches
- ...

Model system - pathway (examples)

- Flavonoid biosynthesis:
 - Anthocyanins: visible as pigments in the flowers of many plant species
 - Proanthocyanidins (PAs): visible as pigments of the seed coat that are responsible for the dark coloration
- Carotenoid biosynthesis
- Betalain biosynthesis (only in Caryophyllales)



Anthocyanin-pigmented flower



Proanthocyanidin-pigmented seeds



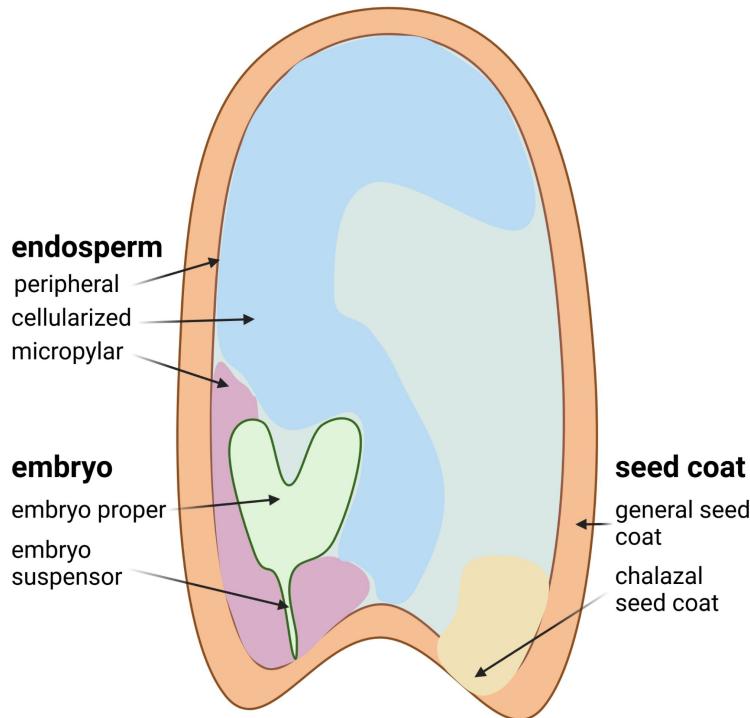
Carotenoid-pigmented carrots



Betalain-pigmented flowers

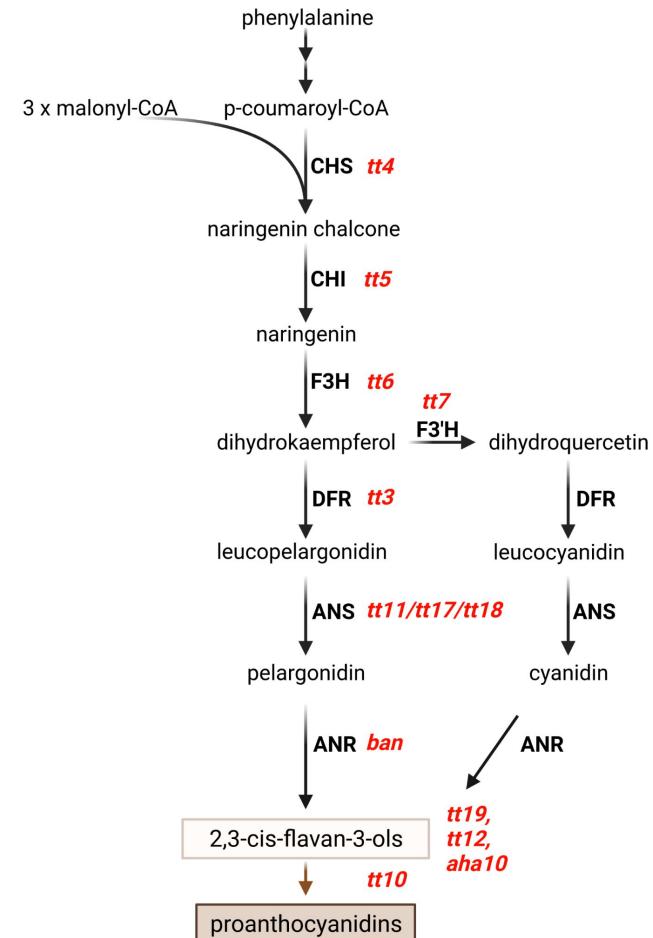
Transparent testa

- Brown proanthocyanidins are located in the testa
- Transparent testa = lack of proanthocyanidins (no pigmentation)



Transparent testa mutants

- Large collection of different tt mutants
- Numbering is based on order of discovery
- Checking if new tt mutants are allelic with existing ones

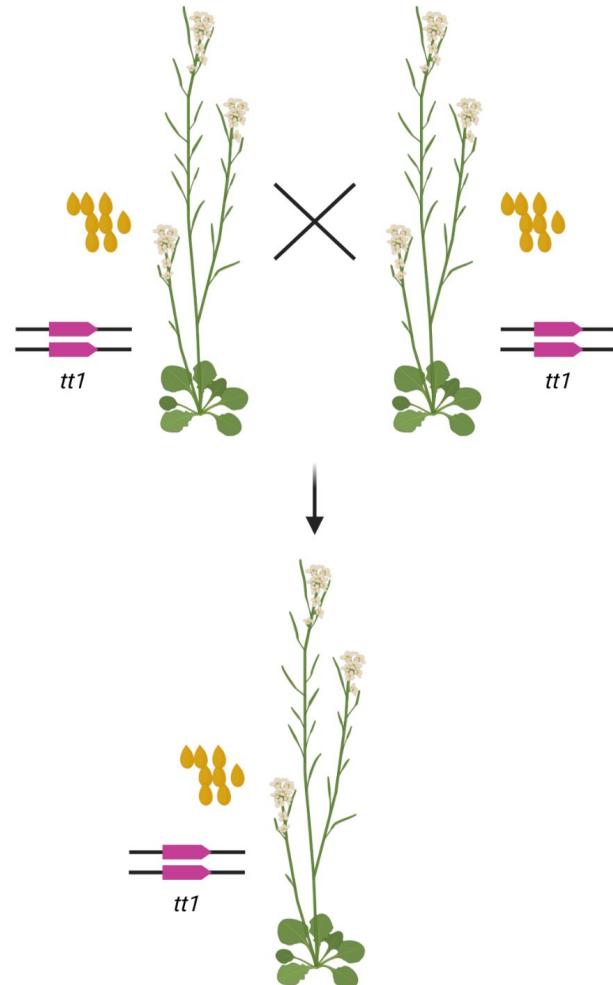


How would you check if two mutations are in the same gene?

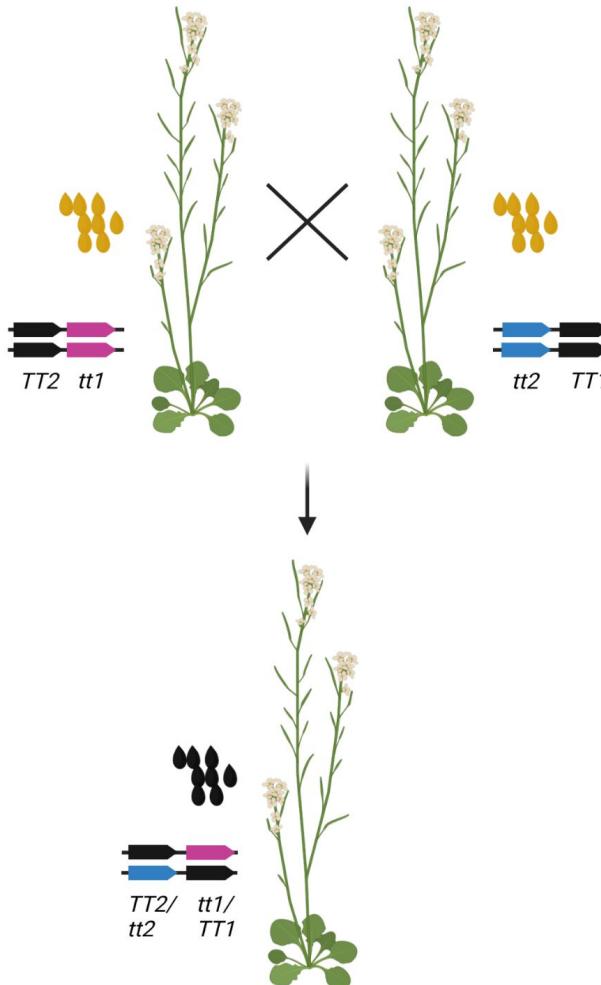


Allelic mutants?

Allelic mutants:

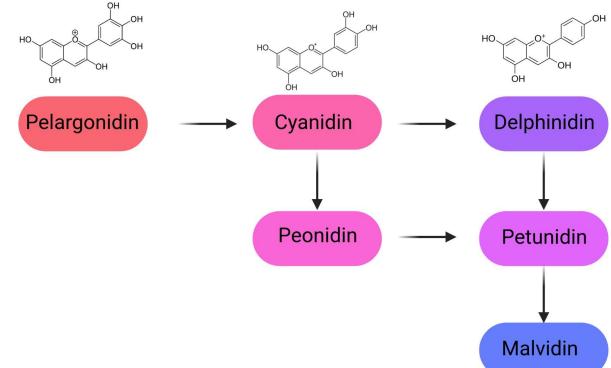


Non-allelic mutants:



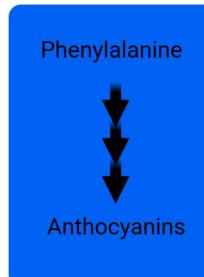
Anthocyanin loss

- Anthocyanins are responsible for red to blue pigmentation of flowers
- Anthocyanins are derivatives from phenylalanine through the flavonoid biosynthesis
- *Digitalis purpurea* is an ornamental plant with biomedical potential
- Different varieties of *D. purpurea* show pigmentation differences

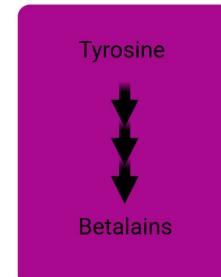


Betalain pigmentation

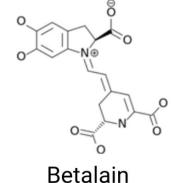
- Betalains are tyrosine-derived pigments
- Production of betalains is restricted to one flowering plant order: Caryophyllales
- Similarities with the anthocyanin biosynthesis e.g. color of pigments
- Evolutionary benefit of betalains over anthocyanins remains unknown



Antirrhinum majus



Mirabilis jalapa



Betalain



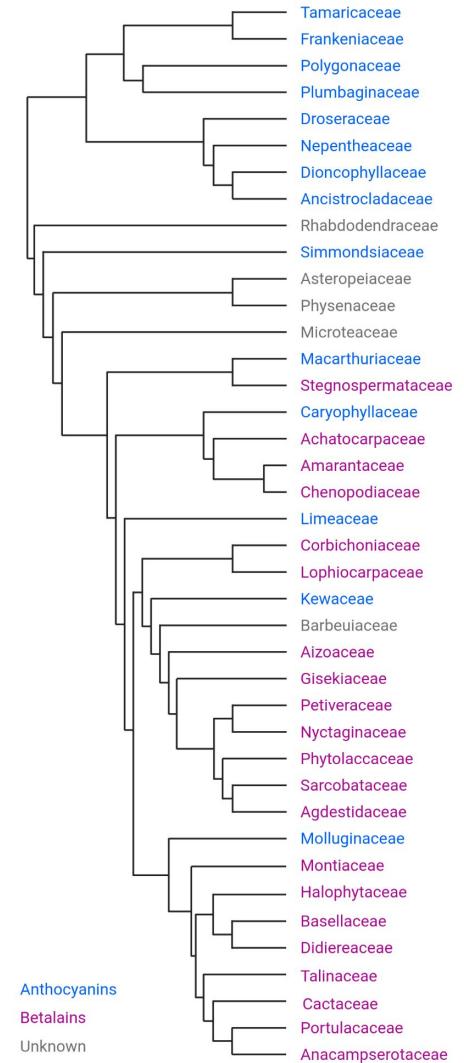
Anthocyanin color range



Betalain color range

Mutual exclusion of anthocyanins and betalains

- Anthocyanins & betalains have similar properties leading to partial redundancy
- Research on anthocyanins & betalains since 1970
- Both pigments have never been observed in the same species
- Mutual exclusion of both pigments is assumed, but mechanism eluded researchers:
 - Enzyme-encoding genes might be lowly expressed
 - Transcription factor(s) activating on anthocyanin biosynthesis genes might have been co-opted for betalain biosynthesis



Brockington et al., 2011/2015: 10.1111/nph.13441

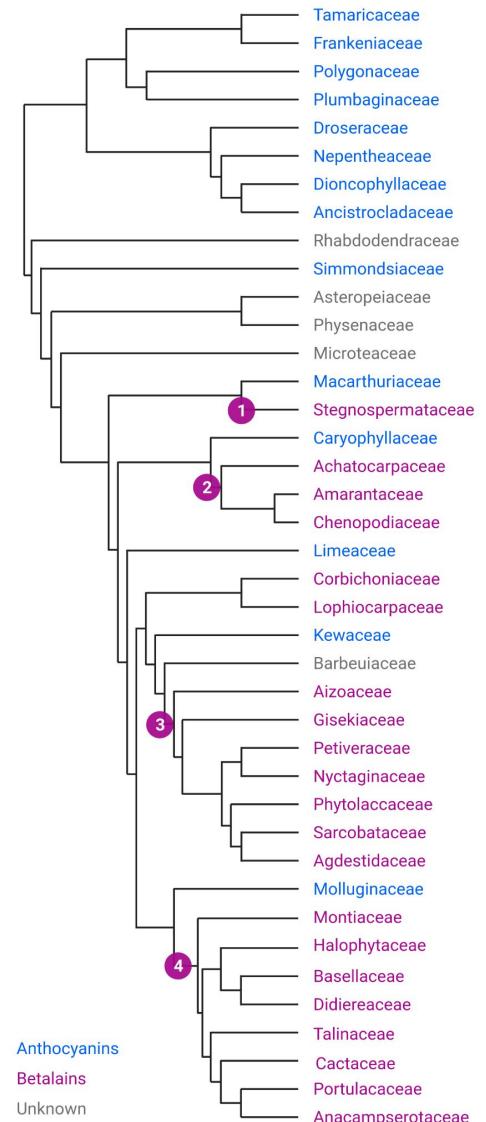
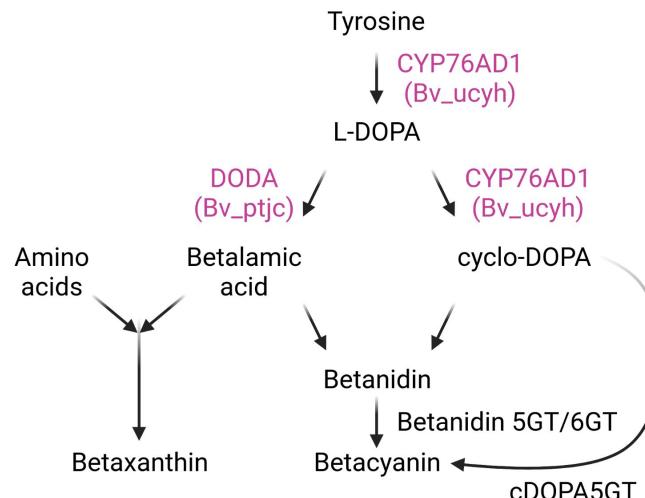
Timoneda et al., 2019: 10.1111/nph.15980

Hatlestad et al., 2015: 10.1038/ng.3163



Independent origins

- Betalain biosynthesis evolved four times independently
- Excellent model system for the investigation of pigment biosynthesis pathways
- Comparison of different betalain origins

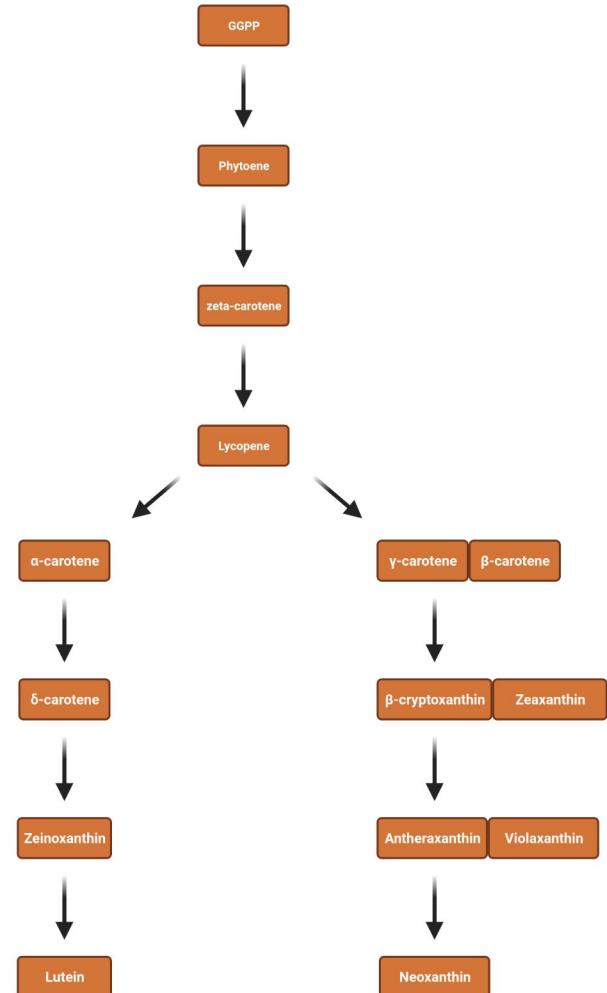


Sheehan et al., 2020: 10.1111/nph.16089



Model system - pathway: Carotenoid biosynthesis

- Carotenoids protect chlorophyll
- Almost all plants have chlorophyll and require this protection
- Are there plants without carotenoids?

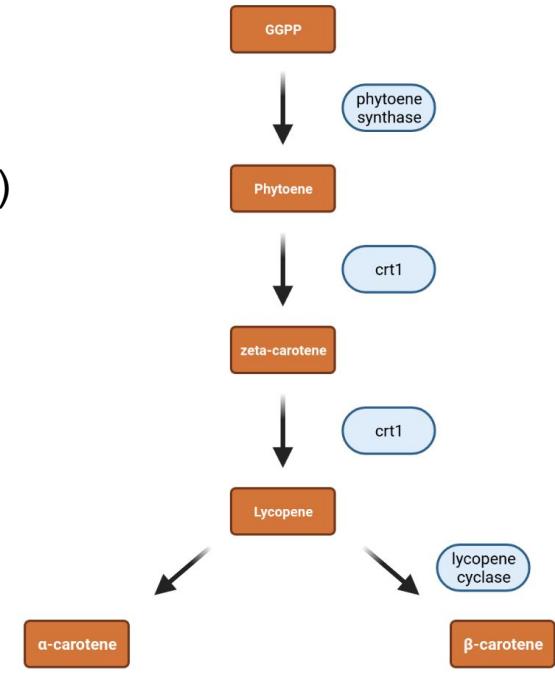


Why are carotenoids not a perfect model system?



Reconstruction of pathways in plants (golden rice)

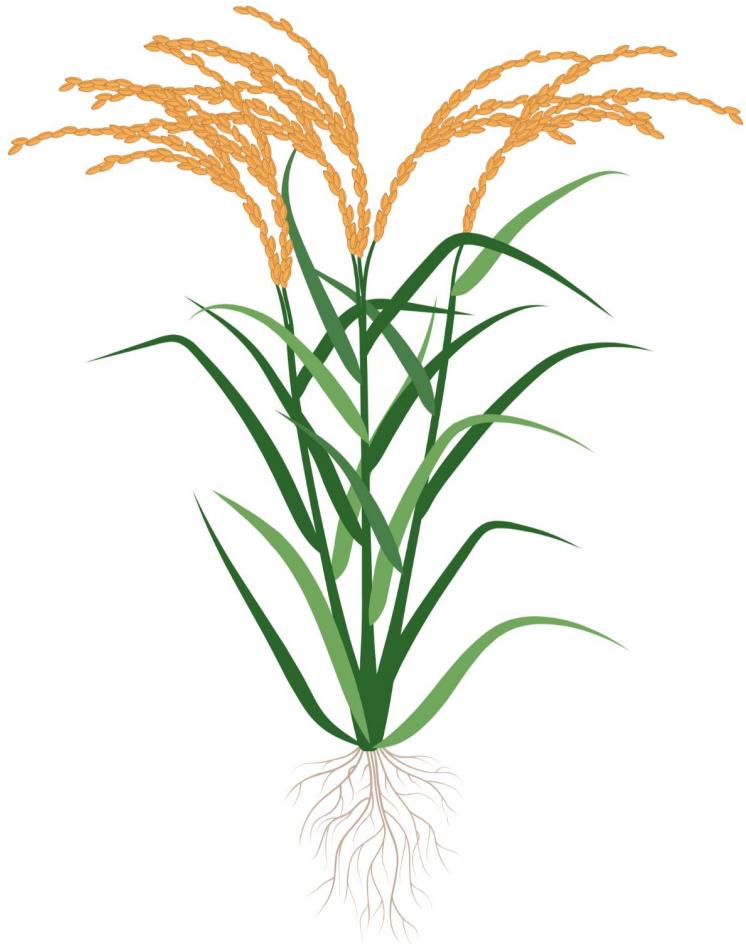
- Vitamin A deficiency causes numerous illnesses (in developing countries)
- Rice was engineered to increase the β -carotene content (provitamin A):
 - psy = phytoene synthase (*Narcissus pseudonarcissus*)
 - crt1 = phytoene desaturase (*Erwinia uredovora*)
 - lcy = leucopene cyclase (already present)



More details about vitamin A: <https://github.com/bpucker/teaching/blob/master/VitaminA.pdf>

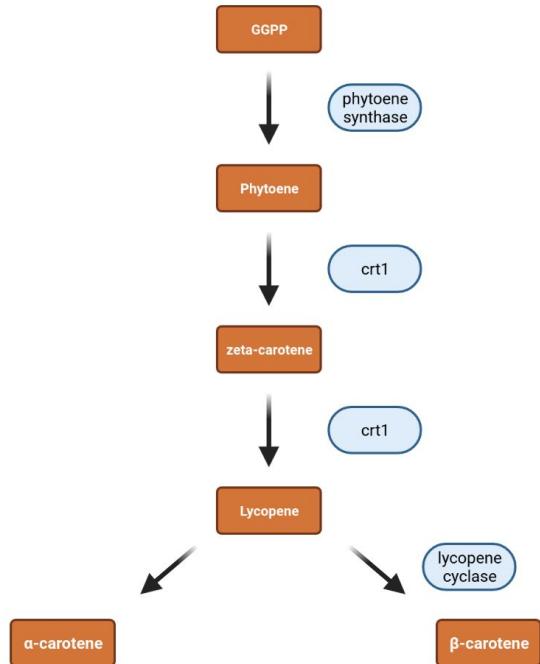
Ye et al., 2000: 10.1126/science.287.5451.303
Schaub et al., 2005: 10.1104/pp.104.057927

How to improve golden rice?



Golden Rice 2.0

- General considerations:
 - Optimization of gene expression in tissues (endosperm)
 - Stability of system over time
 - Genomic integration locus should not influence other traits
- Golden Rice 2:
 - Selection of effective enzymes (maize psy is more efficient)
 - >20x increase in carotenoid production



Paine et al., 2005: 10.1038/nbt1082

Changing flower color of petunia

- Discovery of antisense transcripts by expressing *CHS* heterologously in petunia
- Pigmentation was not enhanced, but reduced by RNA-mediated gene silencing (50x reduced *CHS* transcript)

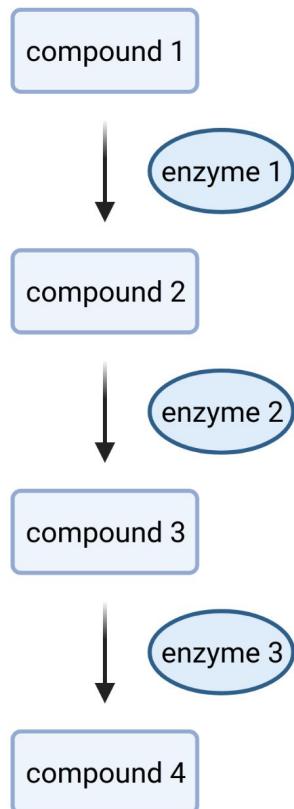


https://commons.wikimedia.org/wiki/File:Petunia_Hybrid_Bi_Color.jpg

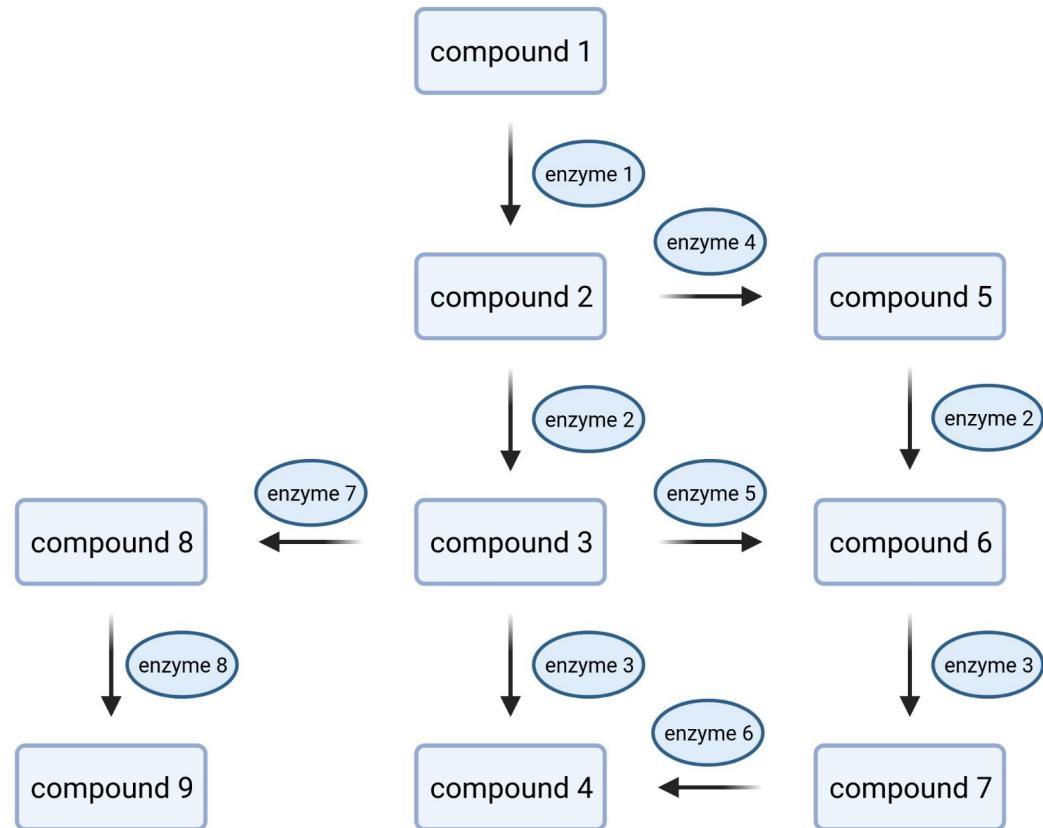
Napoli et al., 1990: 10.1105/tpc.2.4.279
Van der Kroel et al., 1990: 10.1007/BF00027492
Reviewed by Eamens et al., 2008: 10.1104/pp.108.117275

Pathways are networks

Text book

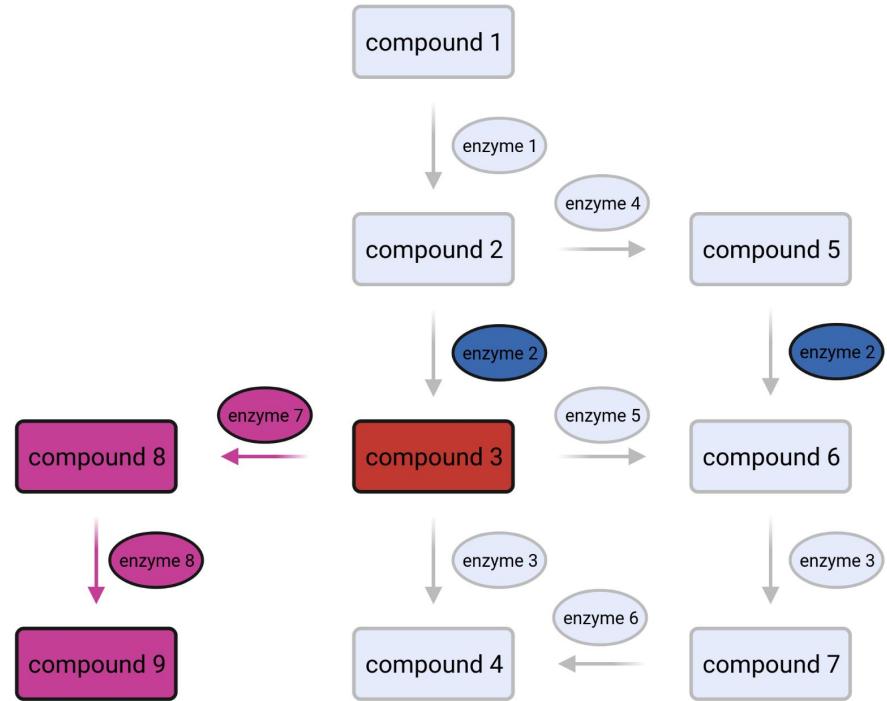


Reality



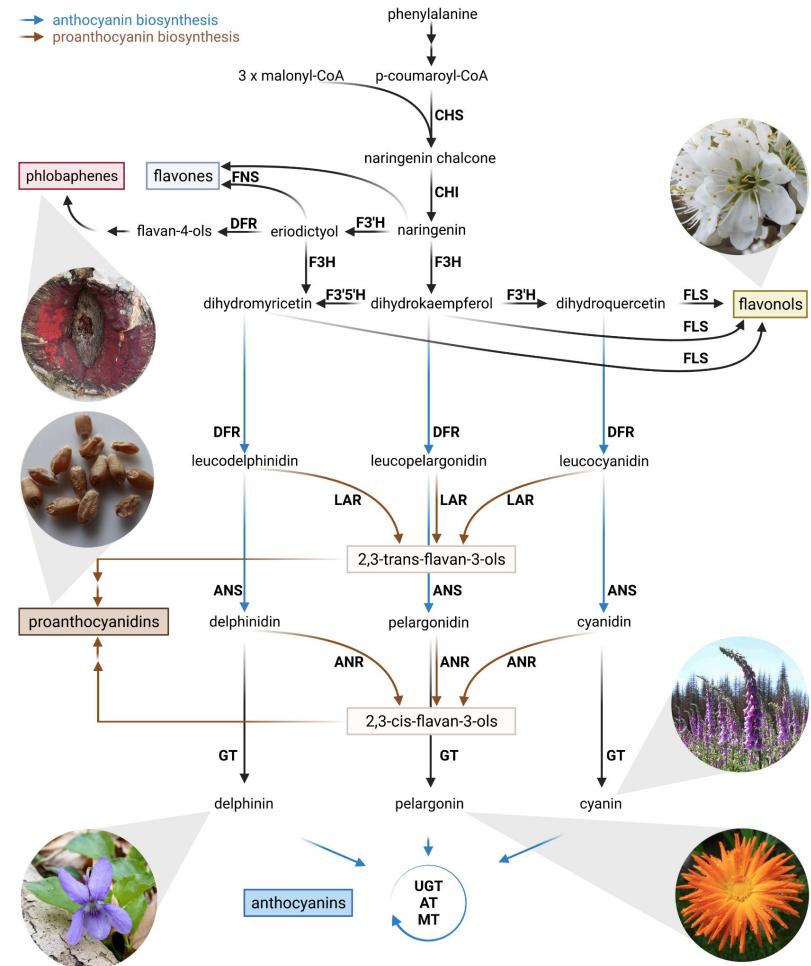
Metabolic networks

- Metabolic networks are characterized by hubs (central intermediates)
- Many enzymes show promiscuity i.e. catalyze different reactions
- Branches could be considered linear pathways



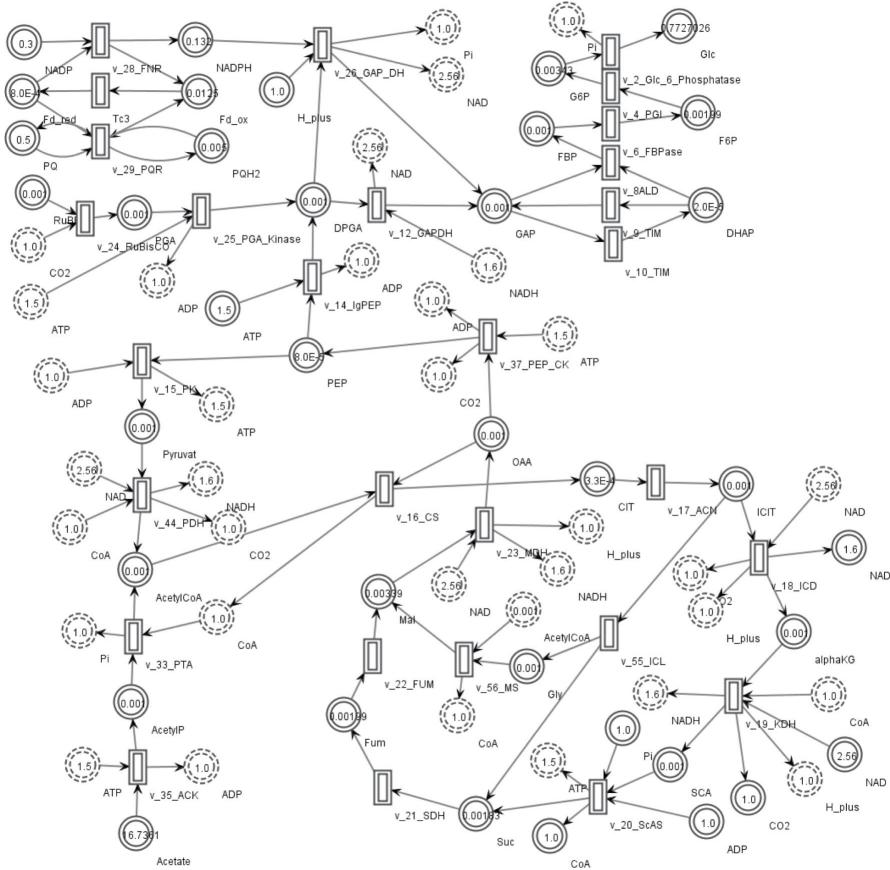
Metabolic networks - example

- Flavonoid biosynthesis:
 - central compounds like naringenin
 - enzymes catalyze different reactions: F3'H, FLS, DFR, ...
 - complex network with different branches: proanthocyanidins (brown), anthocyanins (blue)



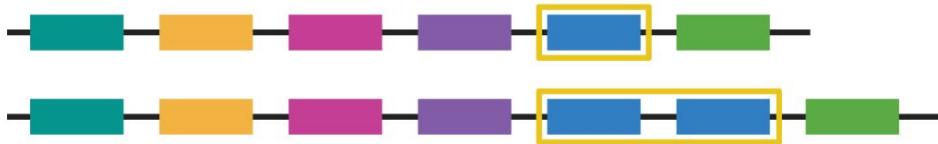
Metabolic network - example 2

- Central carbon metabolism of *C. reinhardtii*
- Well studied system with quantitative information about different enzymatic reactions



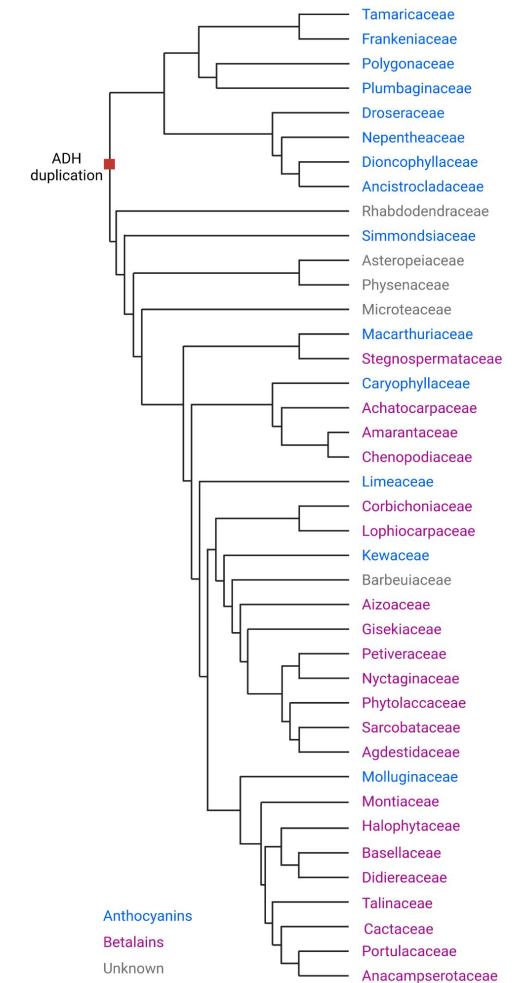
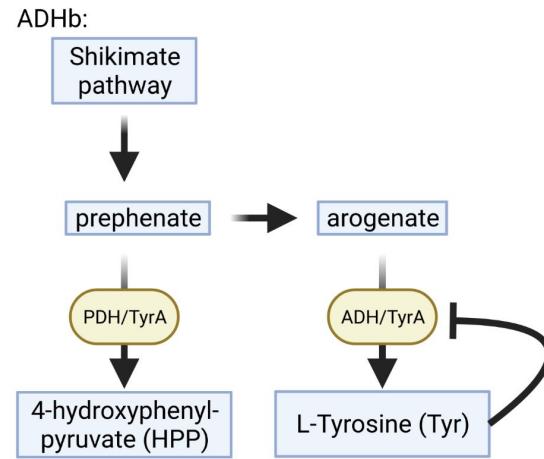
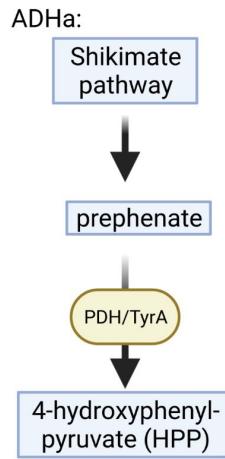
Importance of gene duplications (isoforms)

- Gene duplications form the basis for the evolution of novel genes
- Encoded enzymes can acquire novel functions
- Identification of gene duplications can reveal candidates



Importance of gene duplications (isoforms) - example

- ADH duplication gave rise to feedback-resistant isoform
- ADHa might be required for betalain pigmentation



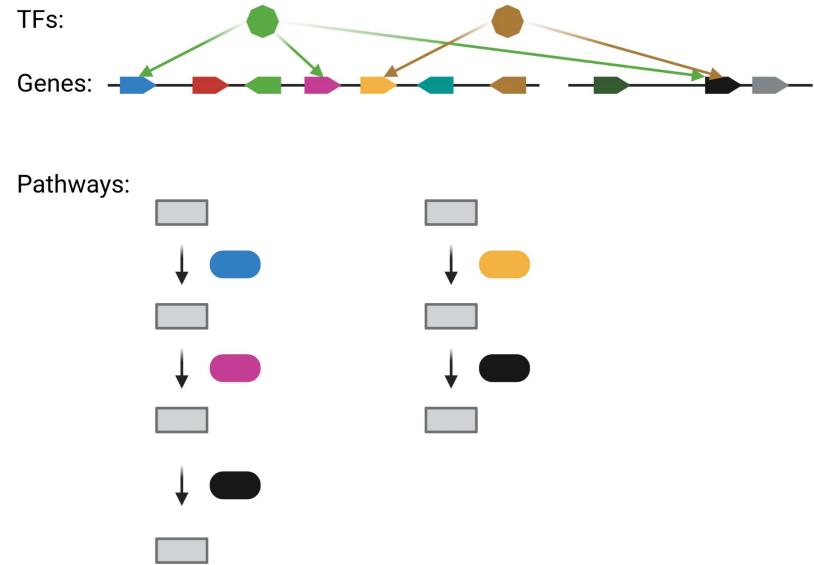
Lopez-Nieves et al., 2017: 10.1111/nph.14822



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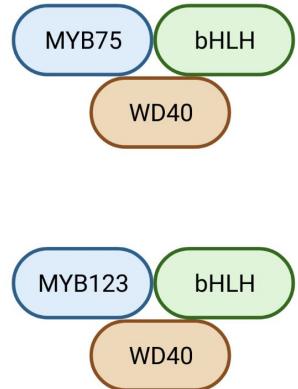
Transcriptional regulation of biosynthesis pathways

- Biosynthesis pathways are controlled by transcriptional regulation
- Transcription factors (TFs) ensure activation of all required genes
- Genes can be involved in different pathways



Transcriptional regulation of the flavonoid biosynthesis

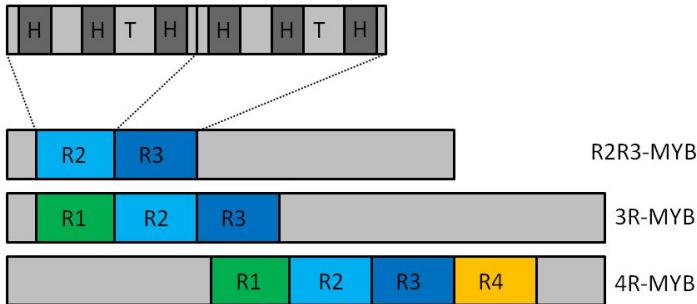
- Flavonoid biosynthesis is controlled by several transcription factor (TF) families
- MYBs are considered specific regulators of different flavonoid biosynthesis branches
- bHLHs are co-activators required for multiple branches
- WD40 (TTG1) is considered a scaffold protein for the connection of MYB and bHLH
- MBW complex: MYB + bHLH + WD40
- WRKYs might be associated with the MBW complex



MYBs

- MYB (myeloblastosis) is one of the largest TF families in plants
- Characterized by repeats (R)
- R2R3-MYBs are particularly important in plants
- Involved in numerous functions in plants

W-(X₁₉)-W-(X₁₉)-W-....-F/I-(X₁₈)-W-(X₁₈)-W-



Based on Dubos et al., 2010

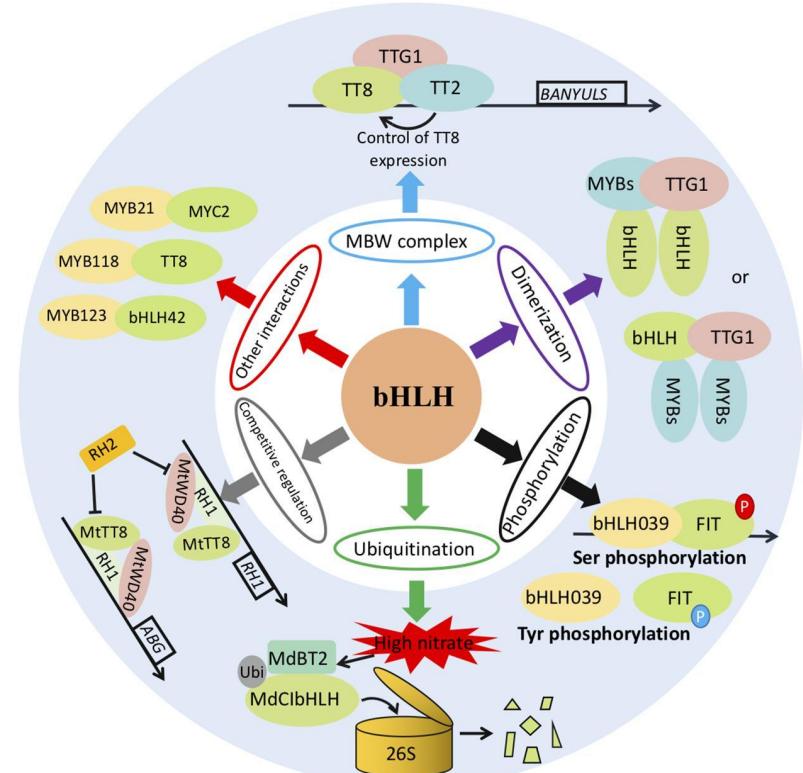
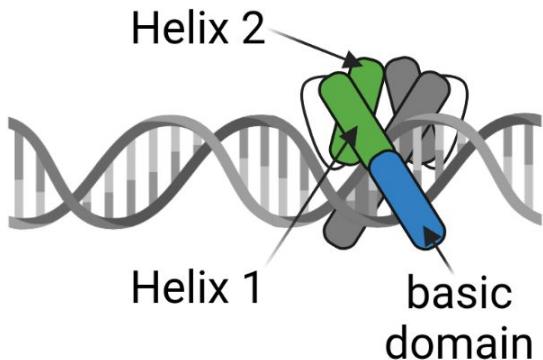


Pucker et al., 2020: 10.1371/journal.pone.0239275
Dubos et al., 2010: 10.1016/j.tplants.2010.06.005



bHLHs

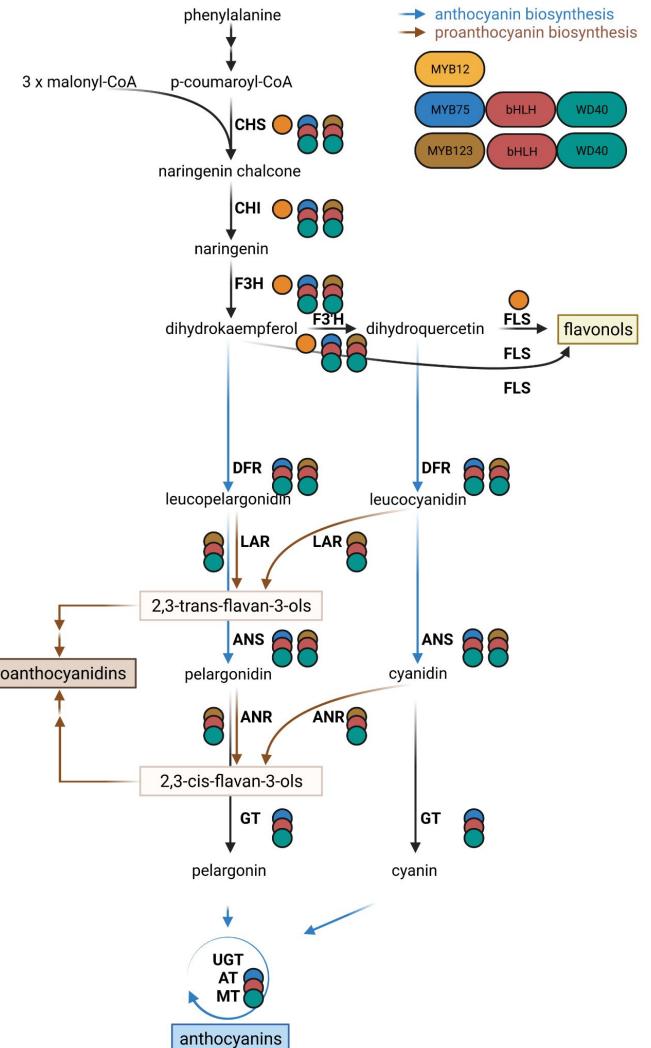
- bHLH (basic Helix-Loop-Helix) transcription factors form a large family in plants
- Transcriptional activation in cooperation with MYBs and independently
- bHLH transcription factors operate often in dimers



Qian et al., 2021: 10.3389/fpls.2021.677611

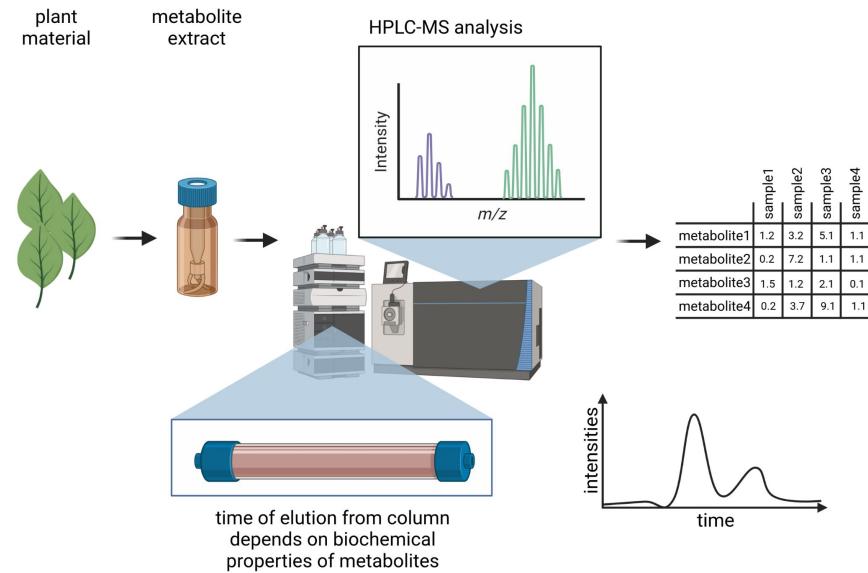
Flavonoid biosynthesis mutants

- Example 1: *fls* or *myb12*
 - Flavonols (-)
 - Anthocyanins (+)
 - Proanthocyanidins (+)
- Example 2: *chs*, *chi*, or *f3h*
 - Flavonols (-)
 - Anthocyanins (-)
 - Proanthocyanidins (-)
- Example 3: *bhlh*, *wd40*, *dfr*
 - Flavonols (+)
 - Anthocyanins (-)
 - Proanthocyanidins (-)

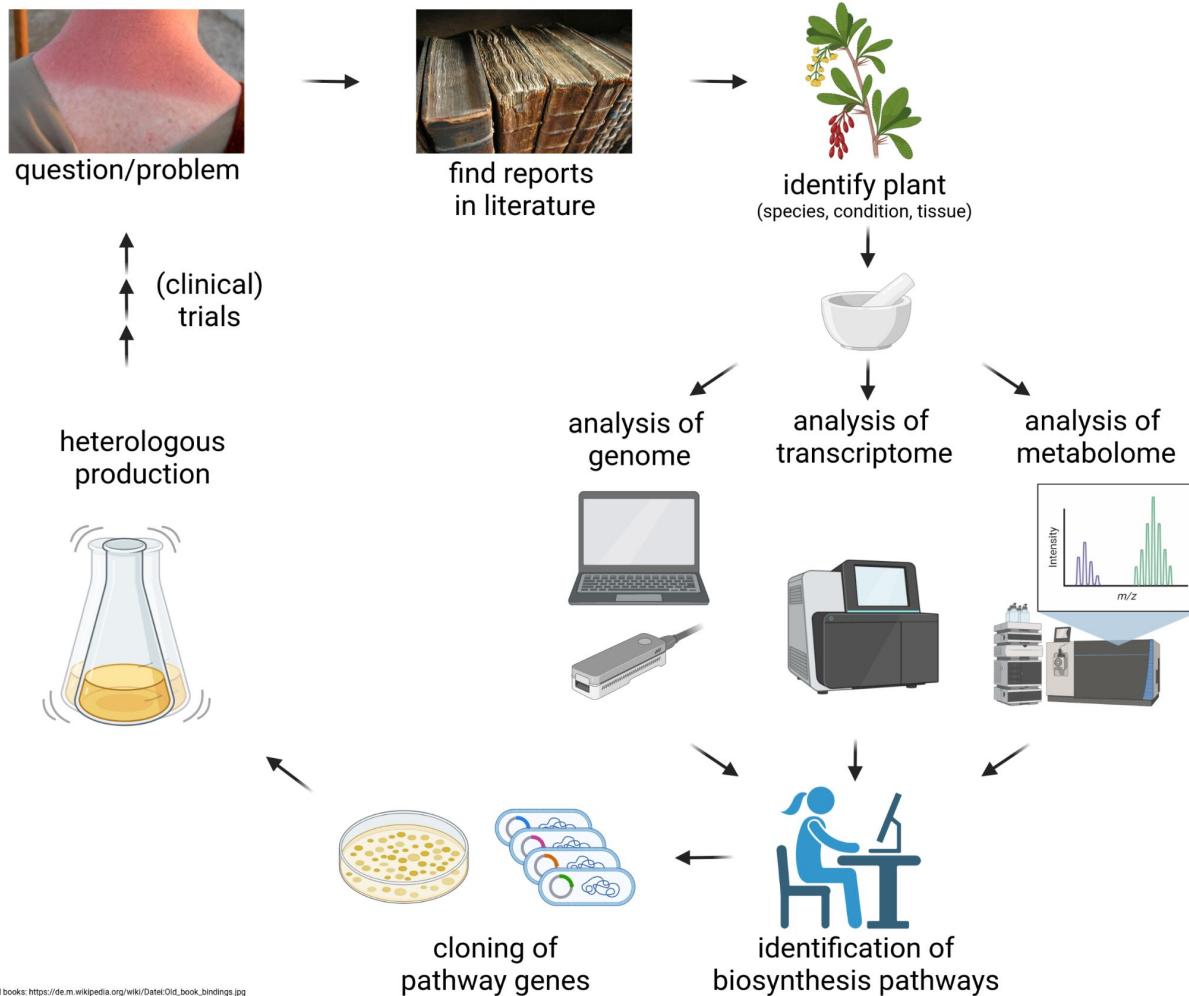


Metabolite analytics

- Discovery of pathways requires quantification of metabolites
- HPLC-MS and GC-MS are standard methods for metabolite quantification
- Integration of metabolite data with other omics



Literature-based pathway search

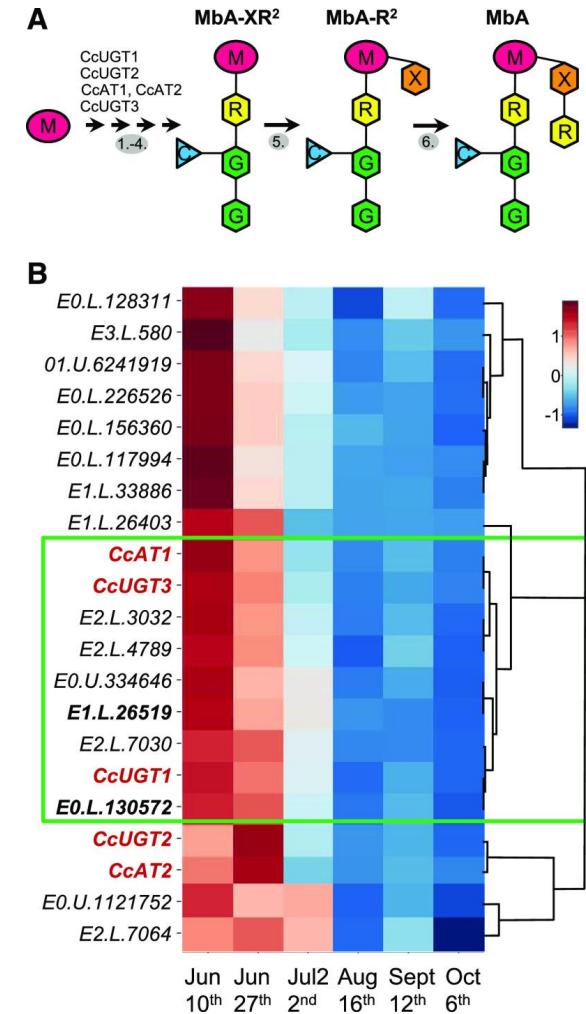


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sun burn: https://en.wikipedia.org/wiki/Sunburn#/media/File:Sunburn_Treatment_Practices.jpg



Discovering a novel biosynthesis pathway: montbretin A

- Identification as inhibitor of α -amylase in screen (enzyme assay)
- Identification of this myricetin-based metabolite in *Crocosmia x crocosmiiflora*
- Search for biosynthesis genes:
 - Co-expression analysis reveals candidate genes
 - Characterization of candidates through assays
 - Reconstitution of pathway through heterologous expression



Irmisch et al., 2020: 10.1104/pp.20.00522



Time for questions!



Questions

1. Which specialized metabolites are produced by plants?
2. How to reveal the function of a gene?
3. What are suitable mutagenesis methods in plants?
4. How to transform plants?
5. What is the difference between forward and reverse genetics?
6. Which characteristics of a model species are important?
7. Which characteristics of a model pathway are important?
8. Which biosynthesis pathways in plants are model systems?
9. Which transcription factors regulate the flavonoid biosynthesis?
10. What are the differences between anthocyanins and betalains?
11. Why is the carotenoid biosynthesis not an ideal model system?

Questions 2

1. Mutations in which genes could explain the loss of red flower pigmentation?
2. Loss of which genes could explain the absence of flavonols?

